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ENUMERATION AND IDENTIFICATION OF HETEROTROPHIC BACTERIA FROM DRINKING WATER

by

James T. Staley
University of Washington
Seattle, Washington 98195

Cooperative Agreement No. CR-807570010

Project Officer

Donald Reasoner
Drinking Water Research Division
Water Engineering Research Laboratory
Cincinnati, Ohio 45268

WATER ENGINEERING RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
CINCINNATI, OHIO 45268

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It was noted in the Seattle treatment and distribution system that prechlorinated source water had higher diversities of heterotrophic bacteria than treated drinking water samples. The predominant types recovered from chlorinated drinking waters of that system were Gram-negative pigmented bacteria. The majority of these could not be readily identified to known genera. Some of these could be placed in the poorly described genus Flavobacterium; however, the great variety of types of pigmented Gram-negative bacteria encountered included many that could not be readily classified in this group without further studies including DNA hybridization.

A number of representatives of known genera were encountered among the isolates from the chlorinated drinking waters. Included were members of the genera Caulobacter, Hyphomonas, Aquaspirillum, Vibrio, Gluconobacter, Azomonas, and Aeromonas.

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American People. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay of its components require a concentrated and integrated attack on the problem.

Research and development is the necessary first step in problem solution, and it involves defining the problem, measuring its impact, and searching for solutions. The Water Engineering Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, to preserve and treat public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research and is a most vital communications link between the researcher and the user community.

One purpose of this investigation was to evaluate alternative procedures to the standard plate count procedure for counting viable heterotrophic bacteria from chlorinated drinking waters. Our study indicates that more dilute media, lower incubation temperatures, and longer incubation times all result in higher recoveries of bacteria. Thus, we recommend changes to improve the standard plate count procedure. These changes would result in improved detection of bacteria that are consumed in chlorinated drinking waters.

Another objective of this study was to identify the predominant heterotrophic bacteria found in two different chlorinated water distribution systems. Our results indicate that many of the most commonly encountered bacteria cannot be currently identified. Nonetheless almost half of these isolates can grow at human body temperatures (i.e., 37°C) and are therefore potential pathogens of humans. We also report the identificaiton of isolates from several genera, which to our knowledge, have not been previously reported from chlorinated drinking water.

Francis T. Mayo
Director
Water Engineering Research
Laboratory

ABSTRACT

Various spread-plating enumeration media and procedures have been tested to determine the method of choice for the enumeration of the highest numbers of heterotrophic bacteria from chlorinated drinking waters. Dilute media, including a caseinate peptone starch medium, a dilute peptone medium and R2A medium provided greater recoveries than the standard plate count medium currently employed. In addition, reduced temperatures of 20°C and prolonged incubation periods of 14 to 28 days resulted in the highest recoveries of heterotrophic bacteria from the two chlorinated distribution waters examined.

It was noted in the Seattle treatment and distribution system that prechlorinated source water had higher diversities of heterotrophic bacteria than treated drinking water samples. The predominant types recovered from chlorinated drinking waters of that system were Gram-negative pigmented bacteria. The majority of these could not be readily identified to known genera. Some of these could be placed in the poorly described genus, Flavobacterium; however, the great variety of types of pigmented Gram-negative bacteria encountered included many that could not be readily classified in this group without further studies including DNA hybridization.

A number of representatives of known genera were encountered among the isolates from the chlorinated drinking waters. Included were members of the genera Caulobacter, Hyphomonas, Aquaspirillum, Vibrio, Gluconobacter, Azomonas, and Aeromonas.

This report was submitted in fulfillment of Cooperative Agreement No. CR-807570010 by James T. Staley under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period October 12, 1980 to April 12, 1983, and work was completed as of June 1, 1984.

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SECTION 1

INTRODUCTION

Water is universally consumed in large quantities by the public. Thus, from a public health perspective, it would be desirable to know the types and numbers of bacteria that are ingested by drinking chlorinated water. Yet numerous investigations have shown that the standard method for enumerating heterotrophic bacteria from drinking waters (i.e., the standard plate count or SPC procedure) frequently provides lower counts of bacteria from drinking waters than other procedures (Clark, 1971; Fiksdal et al., 1982; Reasoner and Geldreich, 1979; Taylor and Geldreich, 1979). Furthermore, there is currently no adequate procedure for the characterization and identification of heterotrophic bacteria from drinking waters.

The objectives of this investigation were two-fold: (a) to evaluate alternative procedures for the enumeration of heterotrophic bacteria from chlorinated water supplies and (b) to develop a procedure that could be used to identify the bacteria that commonly occur in drinking waters.

The City of Seattle water distribution system was used as a test system for the study of enumeration procedures. Various procedures were compared with the SPC procedure to optimize the viable count of heterotrophic bacteria.

The final section of the report contains the results of our investigation to characterize and identify predominant bacteria isolated from chlorinated drinking waters. For this part of the study, about 50 isolates were obtained from the Cincinnati water treatment system and a comparable number were isolated from the Seattle water treatment system. These results are reported in Section 5.

SECTION 2

CONCLUSIONS

A variety of media were tested to determine which provided the highest viable counts of heterotrophic bacteria in chlorinated drinking waters from the City of Seattle water distribution system. The most dilute medium tested, (dilute peptone medium, containing only 0.01% peptone and a vitamin and mineral salts solution) consistently provided the highest recoveries of heterotrophic bacteria from the chlorinated samples tested during this investigation.

The highest viable counts of heterotrophic bacteria from the chlorinated samples were obtained when plates were incubated at 20°C rather than 30°C. Temperature shift experiments in which initial incubation occurred at 20°C for 48 h followed by transfer to 30°C for the remainder of the incubation period usually gave higher counts than those incubated at 30°C. But these counts were always reduced compared with those obtained at 20°C. Preliminary experiments in which winter samples were incubated at 11°C suggested that temperatures even below 20°C might yield higher recovery of heterotrophic bacteria.

Maximum bacterial recoveries were obtained after incubation of chlorinated samples longer than 14 days. Normally, 28 days was sufficient for maximum recovery at 20°C; but some bacteria (such as the strain of pink Hyphomonas sp. encountered occasionally) did not appear until after about 40 days.

Total direct microscopic acridine orange (AO) counts usually decreased (frequently by 50%) following chlorination. This result indicates that chlorination can cause sufficient destruction that some bacteria are no longer detected by this technique.

Oligotrophic to mesotrophic waters such as Lake Chester Morse, the source-water lake studied in this investigation, typically have approximately 10^6 total bacteria (AO) per ml. Our results, which are consistent with those of others, indicate that only about 1% of these can be cultivated on laboratory media. In contrast to oligotrophic lakes, eutrophic lakes contain higher percentages of cultivable bacteria. Thus, the percentage of recoverable bacteria (i.e., viable count/AO count X 100) can be used as an indication of trophic state. By this method, one can conclude that the in-city reservoirs can sometimes be characterized as eutrophic waters. During one survey, three of the four in-city reservoirs sampled showed

recoveries ranging from 27% to 42%, and Lake Youngs (the initial reservoir following the first chlorination) showed a recovery of 11.8%.

The diversity of heterotrophic bacteria was estimated by determining the number of each colony type on an enumeration medium. Diversity was assessed by the Shannon diversity index formula. Chlorinated samples were less diverse than the source water during this investigation. Before chlorination, Lake Chester Morse water had high diversity (Shannon index values ranging from 3.0 to more than 4.0) whereas the final chlorinated samples showed low diversity (diversity index values of 1.08 to 2.81). Though diversity indices were not calculated for Lake Youngs, the plates showed that, like the final chlorinated samples, Lake Youngs exhibited a low diversity. The results suggest that chlorination causes a reduction in the diversity of bacteria from this water supply system. However, the practice of holding treated water in open reservoirs may be responsible for or contribute to a lowered bacterial diversity.

A collection of 102 strains of bacteria was obtained from two municipal drinking water systems in which chlorination was used as the disinfection procedure. Approximately half of the strains were obtained from the City of Seattle water distribution system, and the other half were obtained from the City of Cincinnati water distribution system. Approximately half from each city were isolated during the summer months and the other half during the winter months.

The 102 isolates were characterized and where possible identified to known genera using phenotypic tests. Ten of the isolates were prosthecate bacteria, including representatives of the genera Caulobacter (9 strains) and Hyphomonas (1 strain). To our knowledge, this is the first time in which these bacteria have been reported in chlorinated drinking water, though both genera have been known to occur in freshwater habitats. Among the other morphologically distinctive genera discovered were Aquaspirillum (three strains) and a black pigmented strain of Vibrio, a potential fish pathogen. In addition, three strains of a large ovoid organism were identified as Azomonas. A group of Gram-positive to Gram-variable rods was also isolated. These were nonfermentative organisms, and only one of them was identified -- a strain of Gluconobacter based on its ability to grow at low pH and produce acid from ethanol.

Most of the isolates were Gram-negative rods. Only a few of these were facultative anaerobes, some of which were identified as strains of Aeromonas hydrophila.

Most of the Gram-negative rods were obligately aerobic. Only one was clearly identifiable as a strain of Pseudomonas, though several other strains were motile and non-pigmented and shared many features of the genus.

Many of the Gram-negative rods could be superficially classified in the genus Flavobacterium because of their pigmentation and other features. But great diversity existed in this group, even more so than in the poorly defined genus Flavobacterium. Thus, further studies entailing DNA hybridiza-

tion would be necessary to determine whether any of these resemble known strains of the genus Flavobacterium closely enough to be classified in the genus.

SECTION 3

RECOMMENDATIONS

Development of suitable procedure for viable enumeration of heterotrophic bacteria

This investigation as well as others have shown the inefficacy of the SPC procedure for the recovery of heterotrophic bacteria in chlorinated drinking waters. More dilute media, lower incubation temperatures, and longer incubation times are necessary for optimum recovery of bacteria from some chlorinated drinking waters. In this investigation, we report that the most dilute medium, DP agar, is most effective at growing heterotrophic bacteria. This medium should be compared with other media using disinfected waters from other water supplies. The effect of temperature and length of incubation should also be investigated. The ultimate goal would be to develop a more suitable medium for assessment of heterotrophic bacterial numbers from a wide variety of sources. Different geographic regions in the United States may require somewhat different enumeration procedures because of water type, treatment type, annual temperature ranges, etc.

Development of an alternative rapid technique for enumeration of heterotrophic bacteria from drinking waters

Viable plating procedures will never provide rapid estimates of bacterial numbers because of the long incubation times required. Thus EPA should examine alternative approaches for the rapid enumeration of heterotrophic bacteria. The most promising approach at this time would be to use the INT staining procedure. Effective chlorination should result in a low ratio of the INT microscopic count of respiring organisms to that of the total AO count. If research bears this out, these two procedures, which can be handled simultaneously, could be used. Both can be performed rapidly in the laboratory. It is also possible that these techniques might be ultimately converted into simpler and less tedious colorimetric or fluorometric assays. For example, the INT procedure is adapted from a colorimetric assay for election transport activity, the ETS assay (Christensen and Packard, 1979).

Determination of why the diversity effect occurs in the Seattle drinking water system and whether it is common in other treatment systems

Our results have consistently shown that the bacterial composition of

source water is much different and much more diverse than that of the disinfected water. The result may be due to a number of factors, but there is presently no way of knowing why this phenomenon occurs. One hypothesis to explain the decrease in diversity is that it is a result of chlorination. The chlorination treatment may kill most of the bacteria and permit the survival of only a limited number of species, and these thrive in the downstream reservoirs. But the high numbers of bacteria in Lake Youngs and the in-city reservoirs indicate only that active regrowth is occurring in the reservoirs after chlorination. Since these reservoirs no longer have residual chlorine in them, another explanation for the decreased diversity is that conditions in the reservoirs favor the growth of a limited number of bacterial species. One could argue that since these reservoirs are exposed to light, selected, pigmented species survive chlorination, and they have established themselves over a long period of time. Thus the bacteria in the open reservoirs are not those that survive chlorination but those that are able to grow best after the chlorination. One could also argue that because of chlorination, the nutrient composition of organic or inorganic materials has been changed and the change favors regrowth of selected bacterial species. Algae blooms and dust entering open reservoirs may also contribute to conditions that would favor the growth of selected bacterial species.

Experiments could be designed to test these various hypotheses. For example, if the reduction in species diversity occurs at the time of chlorination, then sampling of waters following the first chlorination should already show a reduction in diversity.

The diversity reductions noted in this investigation are among the most dramatic reported for bacteria in the literature. If the reduction occurs at the point of chlorination, it might be possible to develop a test to assess the effectiveness of chlorination by measuring diversity, as well as numbers of survivors, though it may not be possible to perform this type of test very quickly.

The need to know the identity of the common bacteria encountered in drinking waters (particularly pigmented strains that grow at 37°C and other potential pathogens)

This investigation shows that many of the most common bacteria from the two drinking water systems cannot be readily identified to known bacterial taxa. Since these organisms are being ingested in large quantities by the public, their identity is important. The most common group of organisms encountered in this study are obligately aerobic, Gram-negative, pigmented rods. Because they are difficult to grow and show little activity by usual nutritional tests, they are difficult to characterize. Furthermore, although they could be placed in the genus Flavobacterium, such action would not clarify their identity, because Flavobacterium is such a heterogeneous collection of different bacteria. DNA hybridization techniques would permit a determination of whether the drinking water strains bear any relationship to those already classified in this genus. This taxonomic question is most appropriately answered by EPA because these organisms appear to be the predominant type of bacterium in chlorinated drinking waters, and they are not known to predominate in any other habitat.

Conceivably, some of these bacteria, particularly those that grow at 37°C and those in genera that contain known pathogens (e.g., Vibrio sp.) may be pathogenic organisms. Thus it is especially important to identify them.

The identification scheme proposed in this study includes the morphological characterization of the bacterial isolates as the first step. Since many operators at treatment facilities would not be trained to identify morphologically distinctive bacteria, some training might be necessary. EPA could also provide standard bacterial preparations to aid the identification of the morphologically identifiable genera. Although the phase contrast microscope was used for this investigation, organisms such as Caulobacter spp. can be identified when stained and observed in the ordinary brightfield microscope. Thus, EPA could supply stained slides of Caulobacter, Hyphomonas, Aquaspirillum, etc. for use in identification.

The other tests used in this study are simple physiological tests that require a minimum of equipment and materials. For example, anaerobic growth is accomplished using a simple BBL Gas Pak container (Baltimore Biological Laboratories, Baltimore, MD). With appropriate control organisms such as a Pseudomonas sp. as an aerobe, and E. coli as a facultative anaerobe, there should be no difficulty in determining whether an unknown organism can grow anaerobically.

SECTION 4

Enumeration of Viable Heterotrophic Bacteria from Chlorinated Drinking Water

Sites, Materials and Procedures

Description of Seattle water distribution system (SWDS). Seattle receives water from the Cedar River and the south fork of the Tolt River. Both watersheds drain westerly from the Cascade Mountains in the eastern part of King county (Figure 1). These two rivers furnish high quality mountain water of unusual clarity that requires minimal treatment. The Cedar River system which serves the southern and central area of Seattle has been in operation since 1901 while the Tolt River supply started in 1964. Because most analyses were conducted on Cedar River samples, the Cedar River system is considered in more detail below.

Cedar River Conveyance System.

The Cedar River watershed consists of 37,000 hectares ranging in elevation from 1676 m above sea level at the crest of the Cascade Mountains to 160 m above sea level toward the westerly end (Figure 1). More than 80% of the forested area is owned by the City to which it provides revenue from timber harvesting. The Cedar River in its course flows through Chester Morse reservoir which is about 0.9 km in width, 8 km in length, approximately 30 m deep and has a 50 day detention time. Subsequently the Cedar River is joined by Taylor Creek and flows to the low concrete diversion dam at Landsburg, 24 km below the reservoir dam. At this point the river has an average flow of 1630 million liters daily. At the headhouse (Figure 2) the 425 million liters water that is diverted daily is screened, chlorinated (Table 1) and fluoridated. It then flows through a 4 km long 244 cm concrete conduit. At the lower end of the conduit tunnel, two 198 cm pipelines carry the water an additional 10 km to the 295 hectare Lake Youngs which is surrounded by 830 hectare of protected watershed at an elevation of 150 m. The 16,500 million liter reservoir provides the primary storage for the city and allows for some sedimentation of the turbidity causing particles. It also contains a 30-40 day supply of water which is utilized when the diversion is stopped during periods when turbidity in the river exceeds 3 NTU. Lake Youngs is sometimes by-passed during the summer when algal growth imparts a slight odor to the water.

Water from Lake Youngs is chlorinated as it leaves the lake. It travels 2.4 km through a 244 cm concrete tunnel to the control works where it is piped into four mains of 130 cm to 183 cm diameter and conveyed for 25 to 50 miles to the different parts of the city. It is stored in in-city

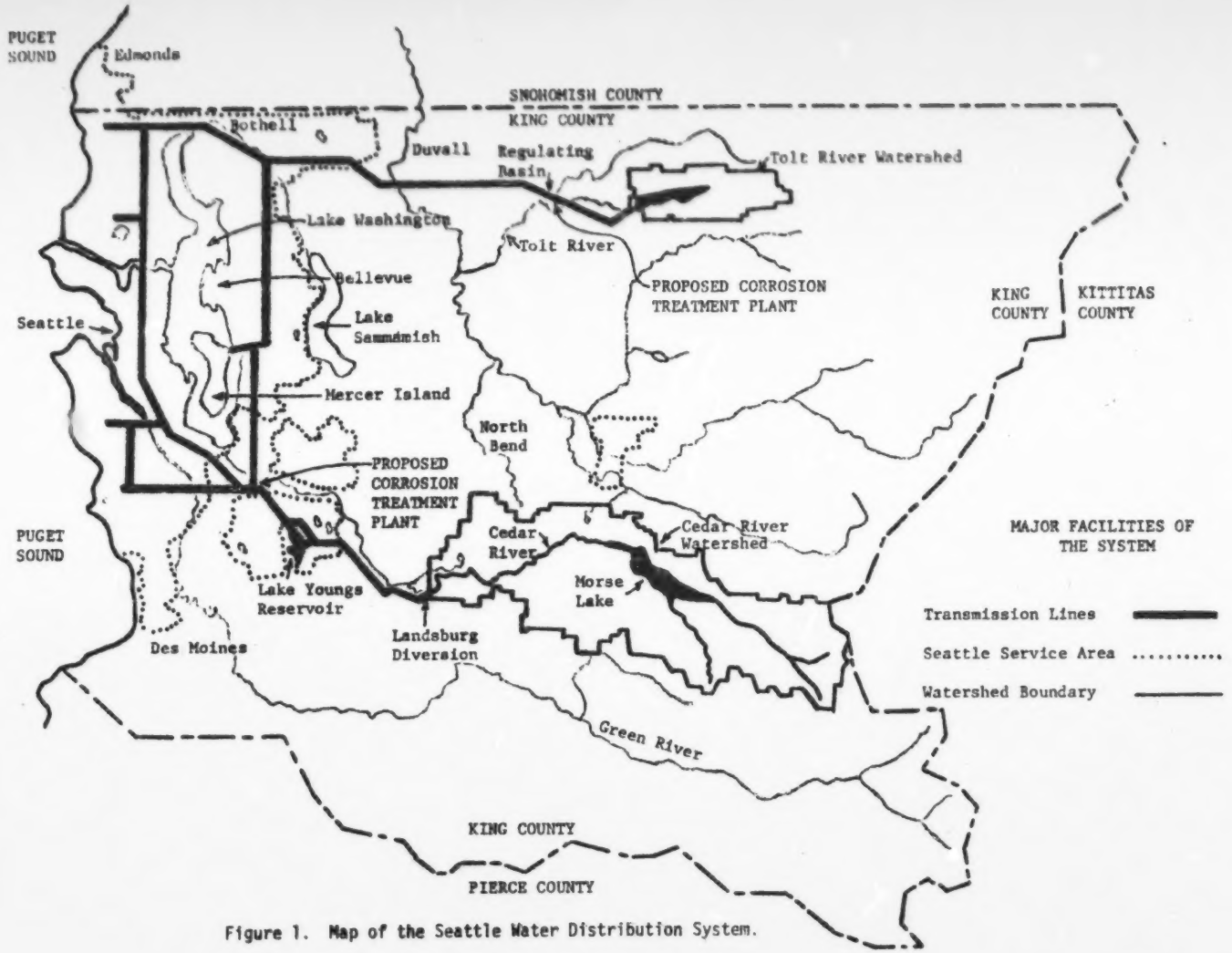

Figure 1. Map of the Seattle Water Distribution System.

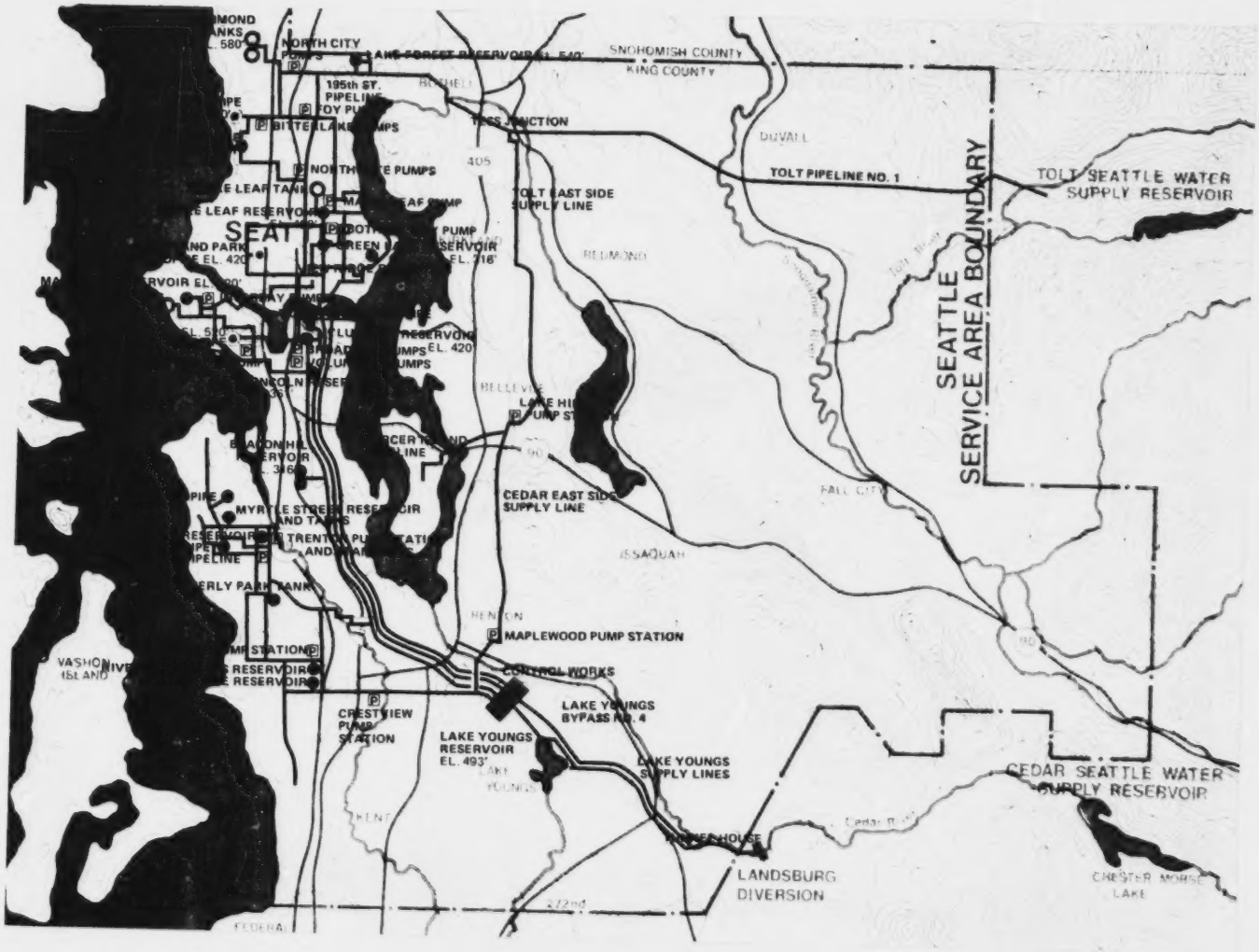

Figure 2. Location of Supply Lines and Reservoirs.

TABLE 1. CHLORINE DOSAGES USED AT THE DIFFERENT CHLORINATION POINTS (1981)

Chlorine Dosage (mg/l)	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Detention Time in Jan. and July(Days)
Cedar River at Lansburg	1.00	0.92	0.99	1.04	1.01	1.06	1.00	0.96	1.02	1.04	1.05	1.01	----
Cedar River at Youngs	0.90	0.90	0.91	1.10	0.90	0.92	1.07	1.19	1.22	1.13	1.17	1.25	41.9/30.6
Beacon	0.65	0.63	0.64	0.63	0.62	0.66	0.59	0.60	0.60	0.65	0.66	0.63	11.9/8.4
Bitter Lake	0.81	0.79	0.79	----	----	----	0.84	0.78	0.68	----	----	----	18.5/32.4
Green Lake	0.63	0.62	0.70	0.75	0.70	0.70	0.75	0.70	0.69	0.69	0.67	0.66	10.8/9.7
Lake Forest	----	----	----	----	----	0.67	0.68	0.70	0.67	0.71	0.63	0.63	----/4.1
Lincoln	0.59	0.62	0.60	0.68	0.61	0.62	0.60	0.61	0.62	0.61	0.56	0.46	2.0/1.6
Magnolia	----	----	----	----	----	2.93	3.06	2.47	2.87	3.16	----	----	----/2.9
Maple Leaf	0.78	0.84	0.85	0.67	0.65	0.71	0.66	0.66	0.95	0.67	0.77	0.75	19.6/16.9
Myrtle	0.62	0.63	0.63	----	0.67	0.61	0.69	0.73	0.78	0.77	0.64	0.66	4.1/5.2
Volunteer	0.48	0.52	0.53	0.51	0.54	0.62	0.58	0.64	0.58	0.53	0.60	0.58	4.8/2.8
SW Spokane	----	----	----	----	----	----	----	----	----	----	----	----	----
Trenton/ W. Seattle	0.59	0.57	0.60	0.64	0.63	0.61	0.63	0.68	0.68	0.68	0.62	0.53	4.6/4.5

reservoirs where the detention time ranges from 2 to 20 days as listed in Table 1. After leaving the open reservoirs the water is chlorinated for a third time such that a minimal residual in taps at the extreme end of the distribution system is maintained at 0.2 mg/l. Water in one reservoir, i.e., Myrtle, is chlorinated a fourth time as it is fed with water coming out of the West Seattle Reservoir. Two of the reservoirs (i.e., Queen Anne Heights: 75 million liters and View Ridge: 9.5 million liters) are covered and therefore do not require a third chlorination.

Water Quality

The water is of very high quality as shown in Table 2 and is generally characterized as very soft. Following Chester Morse Lake and the input of several tributaries the hardness and pH increase slightly. However, after chlorination and fluoridation the pH decreases by 0.3 unit. Because of the relatively low pH of the water and resultant problems with corrosion of components of the distribution system, the city began treating with lime in 1982. The surveys described in this study were largely completed by then. The dissipation of the free chlorine and the subsequent rechlorination in the system is reflected by the gradual increase in chloride ions. The nitrate decrease is a result of some microbial growth in the system. Although the organic content of the water is very low, some tannins and lignins are present which decrease after the chlorination. This could result in smaller molecular fragments that may be available for bacterial growth. The total coliforms in the raw water exceeded 100/100 ml for several years especially during late summer (Table 3). While chlorination reduced these values, some aftergrowth was observed in Lake Youngs (Table 4).

Sample collection. Water samples were collected from the distribution system in collaboration with the City of Seattle Water Department. Sterile Whirlpak bags or Nalgene bottles were used for collection of viable samples. These contained prescribed amounts of sodium thiosulphate to inactivate residual chlorine. Temperature and residual chlorine measurements were made at the site with Hach DPD test kits used to determine total chlorine levels at the time of collection. pH measurements were made either at the time of collection or after returning to the laboratory using water collected without added thiosulphate. Temperatures were also recorded for the samples at the time of collection. Samples for viable enumeration were iced until ready for processing, all of which was completed before the end of an 8 hour day. Chlorinated samples were collected at the earliest sample point following the in-city reservoir that would permit a 10 min chlorine contact time at maximum flow rates.

Table 5 lists the locations from which samples have been collected during the course of this investigation. In subsequent places in the text of this report the code designation used by the City of Seattle has been given for the sample location. For example, "K3" is the chlorinated sample from Volunteer Park reservoir and "Reservoir Pre K3" would be a sample taken prior to chlorination from the Volunteer Park reservoir.

Viable enumeration of heterotrophic bacteria. Water samples were spread with sterile glass rods on agar plates of several media, including

TABLE 2. WATER QUALITY AT DIFFERENT POINTS THROUGHOUT THE SYSTEM

Samples collected: January 24, 1978.

(1) Chester Morse Lake at Crib Dam
(2) Landsburg Forebay, Cedar River
(3) Lake Youngs near Intake
(4) Cedar Distribution at So. Forest Street and Airport Way So.

Results given in milligrams per liter, i.e., part per million, except as noted

	Cedar			
	(1)	(2)	(3)	(4)
Alkalinity, Total (as $CaCO_3$)	8.2	24.0	16.2	13.6
Calcium	3.1	8.2	7.4	7.4
Chloride	0.8	0.8	1.9	3.9
Color, standard units	17	12	12	12
Fluoride	<0.1	<0.1	0.96	0.94
Hardness (as $CaCO_3$)	8.6	23.6	20.0	19.8
Iron	0.075	0.03	0.065	0.085
Nitrogen - Nitrate (as N)	<0.05	0.18	0.10	0.05
Oxygen, Dissolved	13.8	12.6	12.1	12.3
pH	6.9	7.6	7.3	7.05
Phosphorus, Total Orthophosphate	<0.002	0.005	0.004	0.003
Residue, Total	25.5	46	37.5	37
Specific Conductance, micromhos	25.5	56.5	52	53.5
Sulfate	0.8	1.0	1.45	1.0
Temperature, °C	2	5.5	6	6.5
Turbidity, NTU	1.7	0.6	0.9	1.4
Tannin-Lignin	0.22	0.20	0.07	0.04

TABLE 3. TOTAL COLIFORM DENSITIES (No./100 ml) IN UNTREATED CEDAR RIVER WATER AT LANDSBURG

Year	J	F	M	A	M	J	J	A	S	O	N	D	Avg.
1970	59	20	40	37	78	360	308	229	219	215	125	52	145
1971	49	47	16	17	79	100	403	378	423	283	145	81	168
1972	56	34	10	11	38	130	258	172	360	247	38	14	114
1973	29	20	27	49	70	138	191	242	138	79	50	35	90
1974	65	35	39	42	71	112	178	128	143	106	77	70	84
1975	79	28	28	49	54	113	257	389	198	320	253	107	163
1976	26	27	11	17	23	73	283	78	47	50	40	7	58
1977	24	20	22	19	30	54	94	85	102	18	25	25	44
1978	16	14	[illegible]	0	39	141	287	144	203	68	41	5	68
1979	8	2	8	13	7	4	5	21	67	38	26	39	20
1980	4	4	12	4									

All results are the geometric average number of coliform organisms per 100 ml.

TABLE 4. TOTAL COLIFORM DENSITIES IN RAW AND RECHLORINATED WATER (No./100 ml)

Year	Landsburg (raw)	Lake Youngs Supply Line (treated)	Lake Youngs Intake Tunnel (raw)	Control Works (treated)
1970	145	.08	7.6	.006
1971	168	.23	1.0	.017
1972	114	.83	1.1	.011
1973	90	---	1.8	.025
1974	84	.03	44.2	.013
1975	163	.44	51.0	.085
1976	58	.00	7.0	.004
1977	44	.00	2.7	.000
1978	68	.01	---	.003
1979	20	.01	---	.000

All results are the geometric average number of coliform organisms per 100 ml.

TABLE 5. SAMPLE LOCATIONS USED IN THE SEATTLE WATER DISTRIBUTION SYSTEM

Watershed Source	Sample Location	Code	Description
Cedar River System	Landsburg	CPR1	Prior to chlorination
	Landsburg	CPTO	After first chlorination
	Lake Youngs	--	--
	Lake Youngs after chlorination	CPL2	After second chlorination
	Lincoln Reservoir	Pre I3	--
	Lincoln Reservoir	I3	After third chlorination
	Volunteer Park Reservoir	Pre K3	--
	Volunteer Park Reservoir	K3	After third chlorination
	West Seattle Reservoir	Pre L3	--
	West Seattle Reservoir	L3	After third chlorination
	Myrtle Reservoir	Pre Myr	Water after L3 (i.e. after third chlorination)
	Myrtle Reservoir	Myr	After fourth chlorination
	Beacon North Reservoir	Pre M3	--
	Beacon North Reservoir	M3	After third chlorination
	Riverton Heights Reservoir (covered)	P1	After second chlorination
Tolt River System	Tolt River Reservoir	TPR2	Prior to chlorination
		A4	Chlorinated sample (directly from Tolt Reservoir)
		A1	Chlorinated sample (taken after Richmond Highlands water tanks)
	Bitter Lake Reservoir	Pre B3	--
	Bitter Lake Reservoir	B3	After last chlorination
Mixed Tolt and Cedar Samples	Maple Leaf Reservoir	Pre D2	--
	Maple Leaf Reservoir	D2	After last chlorination
	Green Lake Reservoir	Pre E2	--
	Green Lake Reservoir	E2	After last chlorination

Standard Methods Agar (SMA), Dilute Peptone Agar (DP), Caseinate Peptone Starch Agar (CPS), R2A medium and R3A medium (Table 6). Plates were poured 2 to 4 days in advance and dried prior to packaging and storage for use. Regular size Petri dishes (100 mm diameter) were normally used; however, large dishes (150 mm diameter) were sometimes used where designated. Some SMA plates were pour plates incubated under SPC conditions (i.e., for 48 h at 35°C), whereas all other plates were spread plates routinely incubated at 20°C and enumerated after 2, 7, 14 and 28-30 days. In one survey the effect of temperature was tested at 30°C and 11°C. Dilution blanks contained 0.1% sterile, peptone water as diluent.

TABLE 6. FORMULA FOR SMA AGAR (OTHER MEDIA FORMULAE LISTED IN TABLE 21)

	amount (g/l)
peptone	5.0
glucose	1.0
yeast extract	2.5
agar	15.0

Diversity measurements. Strain diversity was determined by examination of plates of the medium that provided the highest numbers of colony forming units (CFU). A given number of colonies was included from a sample analyzed on a particular date, usually 35 to 50 in total. The colonies were carefully examined with a dissecting microscope and each type noted was considered to be an individual strain. Thus the colony type was used as an indicator of strain type (Staley, 1980; Fiksdal et al., 1982). A value termed the Shannon index was used as an estimate of strain diversity (Shannon and Weaver, 1963).

$$H' = -\sum_{i=1}^{S} n_i/N \; 3.3 \log_{10} n_i/N$$

where H' = diversity index value
N = total number of colonies counted
n_i = number of colonies of one type (i.e., ith strain)
s = number of strains

The Shannon formula takes into consideration not only the variety of types present in a sample, but also the evenness in their distribution.

Direct acridine orange (AO) microscopic counts. Samples were fixed with formaldehyde to a final concentration of 1% at the time of collection. The staining procedure of Hobbie et al. (1977) was used to determine the number of cells per ml. A Zeiss GFL microscope with an epifluorescence attachment was used to visualize stained cells (Staley et al., 1982).

Results and Discussion

Effect of medium composition upon recovery. All four media were used during the first three surveys [i.e., January 7 (Table 7), January 28 (Table 8), and March 19, 1981 (Table 9)]. The highest counts were obtained after 14 to 28 days of incubation depending upon the sample. Though Standard Plate Counts using SMA incubated at 35°C for 48 hours were often as high or higher than other media at that time, they were never as high as the maximum counts obtained on other media incubated for 28 days. Also, the SMA medium incubated at 20°C was almost always inferior to other media used in the surveys. The one exception was from a chlorinated water sample from the Riverton Heights Reservoir (P1) collected on January 7, 1981 (Table 7). In this particular instance although the count was higher on SMA, the coefficient of variation was greater on SMA than DP suggesting that it may not have been the best medium even on that occasion.

The other three media were found to be superior to SMA under conditions of prolonged incubation (i.e., 14-28 days). The R2A medium provided the highest counts for two of the samples from the first survey (Table 7) and one from the second (Table 8). The samples where it gave the highest counts were from one source water (CPR1) and two reservoir samples. The CPS medium, which is similar in composition to the R2A medium, was found to be generally better than R2A for the samples analyzed in the first three surveys. Nine of the samples gave highest counts on the CPS medium. These included one source water sample (i.e., CPR1 on March 19), three reservoir samples, and five chlorinated samples.

The overall best medium was the most dilute medium, DP. Thirteen of the samples gave their highest counts on the DP medium. These included one source water sample (TPR2, on January 28) and four reservoir samples. All of the other eight samples were of chlorinated water.

Caution must be exercised in interpreting results from the enumeration experiments. Though the mean counts are higher on some media, when the large standard deviations are taken into consideration, it is not possible to conclude in any single survey that one of these three media is actually superior to one or more of the other media. Nonetheless it is striking from a numerical standpoint that the DP and CPS media consistently provided high counts from chlorinated samples. For this reason these two media were used in all subsequent surveys (Tables 10-13). SMA was also often used to provide a Standard Plate Count for a comparison with the test media.

Effect of length of incubation time on recovery. Bacterial counts from prechlorinated source water samples were usually quite high after 14 days (Tables 7, 8, and 9). Most chlorinated samples showed 50% to 100% recoveries after 14 days incubation at 20°C. Additional incubation beyond 28-30 days infrequently showed significant increases in CFU in this investigation. One sampling date for which prolonged incubation (40 days at 20°C) resulted in significantly higher counts was September 2, 1981 (Table 11). In this instance some of the in-city reservoirs and their chlorinated waters (I3, L3, and M3) contained a slow growing Hyphomonas-type organism that did not appear on plates until after 28 days. In one chlorinated sample (I3),

TABLE 7. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM SOUTH SEATTLE DRINKING WATER SAMPLES (CEDAR RIVER WATERSHED) JANUARY 7, 1981

Sample	pH	Temp. (°C)	Residual Chlorine (ppm)	Incubation Time (days)	- Number of bacteria/ml - SMA Mean	(± 2σ)[1]	DP Mean	(± 2σ)	CPS Mean	(± 2σ)	R2A Mean	(± 2σ)
CPR1 (Source)	6.7	-[4]	0	2	61(10)[2]	13(0)	140	170	240	80	910	370
				7	140	95	1000	310	1200	350	1700	790
				14	140	93	1400	400	1400	540	1800	930
				28	140	93	1500	310	1500	570	1800	930
Reservoir Pre I3	6.5	-	-	2	510(940)	62(180)	100	70	<430	420	1900	570
				7	3500	530	5100	1200	5100	550	4600	3200
				14	3900	830	6700	1100	6200	600	5100	3200
				28	4000	810	7100	1650	6600	480	5400	2900
I3	6.7	-	0.3	2	3(3)	12(12)	<3	0	7	12	3	12
				7	7	12	3	12	20	20	3	12
				14	10	0	23	31	33	23	23	64
				28	10	0	23	31	33	23	27	76
Reservoir Pre K3	6.7	-	-	2	10(50)	28(28)	<10	ND[3]	<9	ND	34	24
				7	6700	1600	17000	8800	17000	13000	22000	3400
				14	7700	1200	17000	9100	28000	30000	ND	ND
				28	8000	1100	18000	9200	ND	ND	ND	ND
K3	6.6	8	0.2	2	6(3)	(12)	<6	ND	6	ND	6	ND
				7	26	92	110	140	130	160	74	24
				14	170	160	590	320	290	84	48	35
				28	220	110	610	360	420	80	61	32
P1	6.6	8	0.5	2	3(3)	12(12)	<3	ND	<3	ND	<3	ND
				7	7	12	<3	ND	17	31	<3	ND
				14	74	46	87	12	66	67	47	83
				28	130	84	100	10	70	53	75	14

[1] ±2 standard deviations of the mean.
[2] Standard plate count at 35°C given in parentheses; all other plates incubated at 20°C.
[3] Not determinable.
[4] Means not measured.
[5] Underlined numbers indicate medium and length of incubation which provided the highest count.

TABLE 8. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM NORTH SEATTLE DRINKING WATER SAMPLES (TOLT AND CEDAR RIVER WATERSHEDS) JANUARY 28, 1981

Sample	pH	Temp. (°C)	Total Residual Chlorine (ppm)	Incubation Time (days)	- Number of Bacteria/ml - SMA Mean	(± 2σ)[1]	DP Mean	(± 2σ)	CPS Mean	(± 2σ)	R2A Mean	(± 2σ)
TPR2 (Source-small plate)	6.4	-4	0	2	50	53	<3	ND[3]	37	31	33	61
				7	420	92	450	150	430	210	490	210
				14	430	80	600	110	630	14	530	370
				28	450	13	690	95[5]	670	56	560	370
TRP2 (Source-large plate)	-	-		2	66	46	20	40	46	46	110	120
				7	710	430	650	340	840	790	920	920
				14	780	490	1300	580	1600	1400	1300	1900
				28	790	500	2000	1500	1600	1100	1400	2100
Reservoir pre B3	-	-		2	200	560	<60	ND	<60	ND	<60	ND
				7	2100	8400	400	1100	740	1200	1700	1200
				14	16000	22000	400	1100	74000	62000	46000	15000
				28	19000	29000	110,000	41000	84000	72000	53000	20000
B3	5.8	8	0.3	2	<3	ND[2]	<3	ND	<3	ND	<3	ND
				7	3	12	<3	ND	<3	ND	<3	ND
				14	13	31	17	23	10	35	13	12
				28	23	12	30	53	10	35	13	ND
A1	6.0	8	trace	2	<6	ND	<6	ND	<6	ND	<6	ND
				7	130	170	<6	ND	<6	ND	<6	ND
				14	5600	4500	6900	4800	6900	9200	7800	4100
				28	6700	4400	15000	10000	15000	16000	ND	ND
A4	6.9	8	0.3	2	<5	ND	<3	ND	<3	ND	<3	ND
				7	<5	ND	<3	ND	<5	ND	<5	ND
				14	130	57	120	100	120	130	140	35
				28	150	85	150	170	200	0	180	23

(continued)

TABLE 8. (continued)

Sample	pH	Temp. (°C)	Total Residual Chlorine (ppm)	Incubation Time (days)	- Number of Bacteria/ml - SMA Mean	(± 2σ)[1]	DP Mean	(± 2σ)	CPS Mean	(± 2σ)	R2A Mean	(± 2σ)
Reservoir pre D2	-	-	-	2	<6	ND[2]	60	240	<6	ND	<6	ND
				7	140	240	140	240	20	40	400	700
				14	6300	1800	24000	1600	20000	7400	25000	6600
				28	8400	2600	25000	2100	27000	4600	25000	6400
D2	6.3	9	0	2	54	100	<6	ND	20	40	80	210
				7	1200	620	300	420	620	470	5900	4700
				14	12000	7600	17000	9100	22000	2300	12000	4300
				28	12000	8200	20000	9300	25000	1700	13000	4000
Reservoir pre E2	-	-	-	2	<6	ND	<6	ND	<6	ND	<6	ND
				7	200	400	<6	ND	140	460	260	240
				14	26000	19000	<6	ND	63000	55000	74000	35000
				28	29000	16000	40000	83000	51000	4200	76000	39000
E2	6.9	8	0	2	86	170	30	28	66	24	40	70
				7	510	470	630	1100	180	210	900	480
				14	18000	8900	630	1100	55000	18000	22000	3700
				28	19000	9200	31000	ND	51000	ND	26000	3600

1 ±2 standard deviations of the mean.
2 All plates incubated at 20°C.
3 Not determinable.
4 Not measured.
5 Underlined numbers indicate medium and length of incubation which provided the highest count.

TABLE 9. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM SOUTH SEATTLE DRINKING WATER SAMPLES (CEDAR RIVER WATERSHED) MARCH 19, 1981[2]

Sample	pH	Temp. (°C)	Total Residual Chlorine (ppm)	Incubation Time (days)	- Number of bacteria/ml - SMA Mean	(± 2σ)[1]	DP Mean	(± 2σ)	CPS Mean	(± 2σ)	R2A Mean	(± 2σ)
CPRI	7.6	-	3	2	130	180	<7	ND[4]	50	170	43	30
				7	390	78	140	35	860	2200	480	130
				14	460	30	390	320	1200	2000	690	190
				28	550	310	540	390	1300	3100	670	150
Reservoir pre I3	7.0	-	-	2	<600	ND[4]	<600	ND	<600	ND	<600	ND
				7	600	2400	<2000	ND	4600	8400	26000	46000
				14	72000	38000	80000	12000	63000	46000	77000	14000
				28	73000	37000	84000	18000	67000	62000	80000	14000
I3	6.8	9.0	0.44	2	<6	ND	<6	ND	<6	ND	<6	ND
				7	6	24	20	56	<20	ND	<20	ND
				14	66	46	6900	510	190	180	560	230
				28	770	920	6900	540	210	620	1200	280
Reservoir pre K3	7.0	-	-	2	<30	ND	<30	ND	<30	ND	<30	ND
				7	70	230	<100	ND	270	610	230	640
				14	170	420	100	0	400	530	230	120
				28	170	420	270	310	530	460	330	310
K3	7.0	10.0	0.42	2	3	7	<3	ND	<3	ND	<30	ND
				7	10	20	7	23	7	12	20	53
				14	27	76	180	83	77	31	160	360
				28	40	53	270	92	97	95	180	370
Reservoir pre L3	7.1	-	-	2	6	24	<6	ND	6	24	6	12
				7	1500	560	130	28	510	120	2700	420
				14	29000	5300	37000	6700	49000	13000	31000	2100
				28	31000	5300	40000	2500	52000	14000	36000	5600

(continued)

TABLE 9. (continued)

Sample	pH	Temp. (°C)	Total Residual Chlorine (ppm)	Incubation Time (days)	- Number of Bacteria/ml - SMA Mean	(± 2σ)	DP Mean	(± 2σ)	CPS Mean	(± 2σ)	R2A Mean	(± 2σ)
L3	7.6	10.4	0.4	2	<0.6	ND	<0.6	ND	<0.6	ND	<0.6	ND
				7	4.6	13	16	20	34	6.2	3	2.8
				14	25	9.2	49	22	32	24	37	25
				28	26	11	76	39	43	33	40	23
Reservoir pre M3	7.1	-	-	2	<300	ND	<300	ND	<300	ND	<300	ND
				7	16000	12000	2300	8100	17000	5300	31000	14000
				14	54000	20000	200000	61000	71000	3500	110000	16000
				28	67000	15000	240000	39000	140000	130000	120000	21000
M3	6.8	10.5	0.5	2	<0.6	ND	<0.6	ND	<0.6	ND	<0.6	ND
				7	0.6	2.4	<2	ND	2.6	2.4	<2	ND
				14	8	14	15	6.2	8	12	13	2.4
				28	11	14	29	6.2	8.6	23	17	8.4
P1	-	10.0	0.6	2	0.6	2.4	<0.6	ND	<0.6	ND	<0.6	ND
				7	1.4	2.4	0.6	2.4	<2	ND	2	7
				14	3.4	2.4	11	12	2.6	6.2	6	7
				28	4.6	2.4	14	8	3.4	3.2	6	7

1 ±2 standard deviations of the mean.
2 Underlined numbers indicate best growth medium; all plates incubated at 20°C.
3 Not measured.
4 Not determinable.

TABLE 10. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM SOUTH SEATTLE DRINKING WATER SAMPLES (CEDAR RIVER WATERSHED) JULY 8, 1981[3]

Sample	pH	Temp. (°C)	Residual Chlorine (ppm)	Incubation Time (days)	SPC[2]	Number of Bacteria/ml — DP Mean	DP (± 2σ)	CPS Mean	CPS (± 2σ)
CPR1	-	-	-	2	-	180	110	420	120
				7		870	420	1100	490
				28		1300	720	1600	520
CPTO	6.7	-	-	2	-	0.6	2.4	2	4
				7		8	11	6.6	10
				28		14	17	20	12
Lake Young	6.7	-	-	2	-	>3000	>3000	>30000	>30000
				7		6700	7000	67000	23000
				28		250000	41000	250000	130000
Lake Young after chlorination	6.8	-	-	2		>3	>3	>3	>3
				7		10	20	40	35
				28		53	50	250	190
Reservoir for I3	7.3	17.0	-	2	-	>600	>2400	600	2400
				7		>600	9600	1400	4600
				28		320000	860000	190000	46000
I3	7.1	17.0	0.70	2	0	>30	>30	>30	>30
				7		>30	>30	>30	>30
				28		220000	85000	170000	65000
Reservoir for K3	7.1	-	-	2	-	>30	>30	>30	>30
				7		530	310	130	120
				28		11000	14000	3600	640
K3	7.4	17.0	0.26	2	-	6	24	6	24
				7		120	80	400	340
				28		7800	3100	4900	1100

(continued)

TABLE 10. (continued)

Sample	pH	Temp. (°C)	Residual Chlorine (ppm)	Incubation Time (days)	SPC[2]	- Number of Bacteria/ml - DP Mean	DP (± 2σ)	CPS Mean	CPS (± 2σ)
Reservoir for L3	6.7	17.0	-	2	-	3000	12000	>3000	>3000
				7		7000	12000	13000	31000
				28		270000	92000	190000	100000
L3	7.0	17.0	0.0	2	0	>3	>3	>3	>3
				7		23	31	50	92
				28		TNTC[1]	TNTC	TNTC	TNTC
Reservoir for M3	6.9	-	-	2	-	>300	>300	>300	>300
				7		>300	>300	>300	1200
				28		88000	10000	77000	18000
M3	7.0	17.0	0.38	2	-	>3	>3	>3	>3
				7		3	12	7	12
				28		TNTC	TNTC	TNTC	TNTC

1 Too numerous to count. In these samples 0.1 ml was plated. Therefore, these samples contained more than 3000 bacteria per ml.
2 Standard plate count.
3 Incubated at 20°C except for SPC; number underlined indicates best recovery medium.

TABLE 11. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM SOUTH SEATTLE DRINKING WATER SAMPLES (CEDAR RIVER WATERSHED) SEPTEMBER 2, 1981[4]

				- Number of Bacteria/ml -				
Sample	pH	Temp. (°C)	Incubation Time (days)	SPC[3] Mean	DP Mean	(± 2σ)	CPS Mean	(± 2σ)
CPR1	6.7	-	3	-	370	310	800	1200
			7		2400	200	3300(2)	1400
			14		3600(1)[1]	-	4600(2)	990
			28		3600(1)	-	5500(1)	-
CPTO	6.3	-	3	-	13	31	30	53
			7		27	61	6100(2)	5300
			14		9900(2)	11000	4500(1)	-
			28		12000(1)	-	4500(1)	-
Lake Young	6.8	-	3	-	<670	-	<670	
			7		19000	20000	29000(2)	82000
			14		77000	56000	56000(1)	-
			28		100000	48000	78000(1)	-
Lake Young after chlorination	6.5	-	3	-	<1	-	<1	-
			7		2.4(5)	8.6	0.4(5)	1.8
			14		39.0(2)	1.4	24.0(3)	7.0
			28		60.0(1)	-	32.0(2)	5.6
Reservoir for I3		17.0	2	58	<3.3	-	53	42
			3		690	410	580	150
			27		890	270	810	0
			14		1100	220	960	46
I3	7.0	17.0	2	0				
			3		<3.3	-	10	0
			7		23	12	10	0
			14		90	40	20	20
			28		210	300	23	31
			40		510	190		

(continued)

TABLE 11. (continued)

Sample	pH	Temp. (°C)	Incubation Time (days)	SPC[3] Mean	- Number of Bacteria/ml - DP Mean	(± 2σ)	CPS Mean	(± 2σ)
Reservoir for K3	6.9	18	2	100				
			3		<330	ND	<330	ND
			7		<330	ND	700	1200
			14		87000	18000	65000	34000
			28		89000	23000	86000	43000
K3	7.0	17	2	0				
			3		110	92	100	80
			7		1000	560	700	380
			14		1600	690	910	570
			28		1700	520	1000	560
Reservoir for L3	7.0	-	2	74				
			3		<330	ND	<330	ND
			7		120000	33000	53000(2)	21000
			14		140000	46000	53000(2)	21000
			28		150000	49000	110000(2)	40000
			40		160000	48000		
L3	6.9	17.0	2	0				
			3		<3.3	ND	<3.3	ND
			7		2300	210	<3.3	ND
			14		2600	250	<3.3	ND
			28		2900	290	3500	2200
			40		3100	400		
Reservoir for M3	7.0	18.0	2	64				
			3		<3300	ND	<3300	ND
			7		65000	99000	47000	42200
			14		310000	85000	55000	14000
			28		390000(2)	14000	330000	240000
			40		400000(2)	72000		
M3	7.0	17.0	2	0				
			3		<330	ND	<330	ND
			7		<330	ND	330	1200
			14		140000(2)	44000	1700	5800
			28		150000(2)	41000	96000	41000
			40		160000	18000		

1 In most instances three plates were counted. Numbers in parentheses indicate numbers of plates in other cases.
2 Not measured.
3 Standard plate count.
4 Incubated at 20°C except for SPC: numbers underlined indicates best recovery medium.
5 Not determinable.

TABLE 12. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM SOUTH SEATTLE DRINKING WATER SAMPLES (CEDAR RIVER SOURCE) NOVEMBER 12, 1981[2]

Sample	pH	Total Residual Chlorine	Water Temperature	Incubation Temperature(°C)	Days Incub.	- Number of Bacteria/ml - DP Mean	(± 2σ)	CPS Mean	(± 2σ)	SPC Mean	(± 2σ)
CPR1	7.32	-[3]	-	20	2	2200	2400	5700	1800		
					7	11000	1300	14000	3000		
					13	13000	2000	14000	3100		
					32	14000	2100	14000	3100		
				20-30	2	1000	4900	3300	1300		
					7	8800	2800	11000	1500		
					13	9500	3500	12000	2400		
					32	9700	3700	12000	2500		
				30	2	1500	630	2900	1600		
					7	3000	1100	4400	2000		
					13	3200	1000	4600	2400		
					32	3300	980	4600	2400		
				35	2	-	-	-	-	26	24
CPTO	7.14	-	-	20	2	<1.0	ND[4]	<1.0	ND		
					7	36	41	6.0(2)	17		
					13	51(2)[1]	88	15(2)	20		
					32	98(1)	-	10(1)	-		
				35	2	-	-	-	-	3.0	6.0
Lake Young (after chlorination)	7.09	-	-	20	2	<1.0	ND	1.4	4.6		
					7	8.0	11	18	4.0		
					13	14	14	20(1)	-		
					32	19(2)	14	20(1)	-		
				35	2	-	-	-	-	0.3	1.2

(continued)

TABLE 12. (continued)

Sample	pH	Total Residual Chlorine	Water Temperature	Incubation Temperature(°C)	Days Incub.	- Number of Bacteria/ml - DP Mean	(±2σ)	CPS Mean	(±2σ)	SPC Mean	(±2σ)
Reservoir I3	7.14	-	12.5	20	2	<3	ND	<3	ND		
					7	<3	ND	7.0	23		
					13	120	87	57	64		
					32	150	100	95(2)	71		
				35	2	-	-	-	-	<0.3	ND
I3	7.10	0.75	12.5	20	2	<1	ND	<1	ND		
					7	0.6	2.4	0.6	2.4		
					13	4.0	5.6	6.0	8.0		
					32	5.0	2.8	9.0(2)	2.8		
				35	2	-	-	-	-	2.0	3.5
Reservoir K3	7.15	-	12.5	20	2	<30	ND	<30	ND		
					7	<30	ND	100	200		
					13	1800	2100	530	120		
					32	3400	3600	2200	1100		
				35	2	-	-	-	-	1.3	3.1
K3	7.13	0.50	13	20	2	<3	-	<3	-		
					7	<3	-	<3			
					13	23	31	17	42		
					32	70(2)	85	60	72		
				35	2	-	-	-	-	<0.3	ND

(continued)

TABLE 12. (continued)

Sample	pH	Total Residual Chlorine	Water Temperature	Incubation Temperature(°C)	Days Incub.	- Number of Bacteria/ml - DP Mean	(± 2σ)	CPS Mean	(± 2σ)	SPC Mean	(± 2σ)
Reservoir M3	7.12	-	12	20	2	<3000	ND	<3000	ND		
					7	<3000	ND	<3000	ND		
					13	370000	23000	250000	400000		
					32	460000	420000	330000	170000		
M3	7.22	0.62	13	20	2	<300	ND	<300	ND		
					7	<300	ND	<300	ND		
					13	150000	22000	90000	17000		
					32	160000	22000	130000	23000		
				20-30	2	<60	ND	<60	ND		
					7	<60	ND	<60	ND		
					13	17000	14000	340	620		
					32	25000	14000	460	620		
				30	2	30	120	<30	ND		
					7	30	120	<30	ND		
					13	3400	1800	700	280		
					32	5900	1600	900	280		
				35	2	-	-	-	-	1.3	2.3

1 Three plates were usually counted for each number provided. If fewer than 3 were counted, the number of plates is given in parenthesis.
2 Number underlined is the best medium for a particular sample.
3 Not measured.
4 Not determinable.

TABLE 13. WATER CHARACTERISTICS AND VIABLE NUMBERS OF BACTERIA FROM SOUTH SEATTLE DRINKING WATER SAMPLES (CEDAR RIVER SOURCE) FEBRUARY 24, 1982[2]

Sample	pH	Total Residual Chlorine	Water Temperature(C°)	Incuba. Temperature(C°)	Days Incub.	Number of bacteria per ml[2] DP mean	DP (±2σ)	CPS mean	CPS (±2σ)	SPC mean	SPC (±2σ)
CPR1	7.20			20°	28	5,800(1)[1]	-5	3,400	1,000		
CPR1				20-->30°	28	4,700(1)	-	2,700	1,400		
CPR1				30°	28	1,000(2)	210[2]	830	300		
CPR1				recommended temp[4]						9.3	7.0
CPT0	6.80			20°	28	15(2)	14	11	12		
CPT0				recommended temp						2.0	4.0
L.Young	7.29			20°	28	91,000(2)	60,000	100,000	30,000		
L.Young				recommended temp						1.0	2.0
L.Young (after chlorination)	7.10			20°	28	11	24	6.6	6.2		
				recommended temp						0.7	1.2
Reservoir I3[6]	7.11			20°	28	66,000	13,000	50,000	17,000		
				11°	28	100,000	25,000	79,000	11,000		
Reservoir I3				recommended temp						<0.3	-
I3	7.20	8.0	6.5	20°	28	43	12	35(2)	42		
I3				20-->30°	28	11	16	17	2.8		
I3				30°	28	18	17	13	13		
I3				11°	28	20	8.0	25	4.6		
I3				recommended temp						<0.3	-
Reservoir K3[6]	7.83			20°	28	130,000	75,000	91,000	64,000		
				recommended temp						1.0	3.5
K3	7.24	7.0	6.5	20°	28	<3.0	-	13	23		
K3				20-->30°	28	<3.0	-	-	-		
K3				30°	28	<1.0(2)	-	<2.0(1)	-		
K3				recommended temp						0.3	1.2

(continued)

TABLE 13. (continued)

Sample	pH	Total Residual Chlorine	Water Temperature(C°)	Incuba. Temperature(C°)	Days Incub.	Number of bacteria per 100 ml mEndo mean	mEndo (±2σ)	mCp mean	mCp (±2σ)	mAc mean	mAc (±2σ)	mA mean	mA (±2σ)
CPR1	7.20			20°	28								
CPR1				20-->30°	28								
CPR1				30°	28								
CPR1				recommended temp[4]		17	19	2.8(5)	2.6	<0.2	-	-	-
CPTO	6.80			20°	28								
CPTO				recommended temp		<0.3	-	<0.3	-	<0.2(5)	-	<0.2(5)	-
L.Young	7.29			20°	28								
L.Young				recommended temp		12	9.2	4.6(5)	4.6	0.2(5)	0.9	<0.2(5)	-
L.Young (after chlorination)	7.10			20°	28								
				recommended temp		<0.3	-	<0.3(4)	-	<0.2(5)	-	<0.2(5)	-
Reservoir I3[6]	7.11			20°	28								
				11°	28								
Reservoir I3				recommended temp		<0.3	-	<0.2(5)	-	<0.2(5)	-	<0.2(5)	-
I3	7.20	8.0	6.5	20°	28								
I3				20-->30°	28								
I3				30°	28								
I3				11°	28								
I3				recommended temp		<0.3	-	<0.2(5)	-	<0.2(5)	-	<0.2(5)	-
Reservoir K3[6]	7.83			20°	28								
				recommended temp		<0.3	-	0.7(5)	2.3	<0.3(4)	-	-	-
K3	7.24	7.0	6.5	20°	28								
K3				20-->30°	28								
K3				30°	28								
K3				recommended temp		<0.3	-	<0.2(5)	-	2.0(4)	1.6	-	-

(continued)

TABLE 13. (continued)

Sample	pH	Total Residual Chlorine	Water Temperature(C°)	Incuba. Temperature(C°)	Days Incub.	Number of bacteria per ml[2] DP mean	DP (±2σ)	CPS mean	CPS (±2σ)	SPC mean	SPC (±2σ)	mEndo mean	mEndo (±2σ)
Reser- voir L3	7.24			20°	28	30	120	30	120				
				11°	28	70	220	<30	ND				
Reservoir L3				recommended temp						<0.3	-	<0.3	-
L3	7.24	5.0	6.5	20°	28	10	14	17	12				
L3				20-->30°	28	67	230	57	180				
L3				30°	28	9.0(2)	8.4	5.4	6.2				
L3				11°	28	30	40	10	20				
L3				recommended temp						<0.3	-	<0.3	-
Reser- voir M3[6]	7.49			20°		140,000	29,000	93,000	53,000				
				recommended temp						73	42	<0.3	-
M3	7.22	8.0	6.5	20°	28	300	1,200	<300	ND				
M3				20-->30°	28	100	200	30	120				
M3				30°	28	3.0	12	7.0	12				
M3				recommended temp				<0.7	ND	<0.7	-	<0.3	-
Reser- voir Myrtle	7.35			20°	28	870	420	1,000	720				
				recommended temp						<0.3	-	<0.3	-
Myrtle	7.35	5.0	7.0	20°	28	30	35	10	20				
Myrtle				20-->30°	28	20	35	5.0	14				
Myrtle				30°	28	20(2)	0	10	28				
Myrtle				recommended temp						<0.3	-	<0.3	-

1 Three plates were usually counted for each number provided. If more or less than 3 were counted, the number of plates is given in parentheses.
2 Number underlined is the best medium for a particular sample.
3 mEndo - total coliforms; mCp - Clostridium perfringens; mAc - Acinetobacter calcoaceticus; mA - Aeromonas hydrophila. There is no data for samples Reservoir L3 and below for mCp, mAc, and mA.
4 The recommended temperature of incubation for the specific method where data is reported.
5 Not determined.
6 Reservoir not in service due to relining of pipes; one less chlorination for the respective drinking water samples.

it accounted for an increase in viable counts from 210 per ml to 510 per ml; thus it comprised over half of the viable organisms. In the other two chlorinated samples (L3 and M3), it accounted for less than 6% of the count.

Effect of incubation temperature on recovery. Although it was known that temperatures of 35°C and higher were not as favorable for maximum recovery as 20°C (Fiksdal et al., 1982), it was not known whether a 30°C incubation temperature was also too high for maximum recovery. This was an important question to answer because of the prolonged period of incubation needed to provide highest recoveries at 20°C. Thus, for the November 12, 1981 survey (Table 12) and the February 24, 1982 survey (Table 13), samples were plated on DP and CPS media, and replicate sets were incubated at 30°C, 20°C (normally used for enumeration), and also at 20°C with a temperature shift to 30°C. The hypothesis behind the temperature shift experiment was that some organisms might be able to grow at 30°C, but could not survive a rapid shift to 30°C at the time of plating when the environmental temperature was 10°C to 20°C. The shift experiment was performed as follows: after plating on the two media used, DP and CPS, some of the replicate plates (three at each dilution) were placed in the 20°C incubator. Following 48 h incubation these were transferred to the 30°C incubator and incubated for the remaining 30 days. Control plates were incubated continuously at 20°C and at 30°C for the entire 32 days.

The results of these surveys are shown in Tables 12 and 13. Highest counts of heterotrophic bacteria from the source water were found on the 20°C plates in the first survey (Table 12). The CPS and DP media provided similar counts of 14,000 bacteria per ml. In contrast, SPC plates had 3 orders of magnitude fewer bacteria (only 26 bacteria per ml). It was interesting to note that the shift plates on both media provided counts that were intermediate between those at 20°C and those at 30°C. This indicates that the rapid transfer of bacteria from the temperature of the natural habitat to 30°C resulted in reduced recovery of the bacteria (i.e., 67% were not recoverable on the CPS medium and 76% were not recoverable on the DP medium). However, only 14% of the bacteria did not grow on CPS after shifting from 20°C to 30°C, and on DP, 31% did not grow after shifting from 20°C to 30°C.

During this survey the viable heterotrophic counts were appreciably below those of previous surveys for most samples. However, one of the reservoirs, the reservoir for M3, had high numbers of bacteria, and the chlorinated sample derived from that provide good data for the temperature comparison. The reservoir for M3 contained 460,000 bacteria per ml on the DP medium and 330,000 per ml on the CPS medium. Chlorination resulted in lower counts at M3. Samples incubated on DP provided the highest counts at all incubation temperatures. At 20°C there were 160,000 bacteria per ml whereas at 30°C there were only 5,900 bacteria per ml. Again, as in the source water, the shift experiment counts were intermediate, i.e., 25,000 per ml.

Several interesting conclusions can be drawn from these results. First, in this chlorinated water supply at the time of sampling, many of the heterotrophic bacteria were either psychrotrophic or psychrophilic; i.e., they had an optimum growth temperature below 30°C and a maximum

temperature also below 30°C. This is consistent with the temperature of the water supply, which was below 15°C. Second, some bacteria in the chlorinated sample were able to grow at 30°C, providing they were first incubated at 20°C on the DP medium for 48 hours. This suggests that given sufficient time at 20°C on the DP medium they could in some unknown manner adapt to subsequent higher temperatures or were able to repair metabolic lesions that would otherwise have been fatal if placed directly at 30°C. It is especially interesting to note that this was not true of the bacteria from the chlorinated sample that were grown on CPS. For some reason the shift did not result in any higher counts than were found when the plates were directly incubated at 30°C (M3, Table 12). This is in contrast to the source waters where both the CPS and DP media yielded higher counts on the temperature shifted plates than on those incubated directly at 30°C. Apparently some added stress occurred to the chlorinated bacteria when they were incubated on the CPS medium, at the higher temperature. Finally this phenomenon appeared to be, at least in part, a species specific phenomenon. For example, one of the distinctive colonial types from this experiment formed a red colony. None of the red colonies were found on the 30°C plates or the plates shifted from 20°C to 30°C. Therefore, this particular species was a psychrotroph or psychrophile. Likewise, another distinctive colony type, a small pink colony (Hyphomonas sp) showed the same pattern, i.e., it did not appear on the plates shifted to or initially incubated at 30°C.

The second temperature shift experiment was performed on February 24, 1982 (Table 13). Unfortunately chlorine levels were unexpectedly high and the bacterial counts were correspondingly very low. Also, it should be noted that the city was relining pipes and several of the reservoirs (Pre I3, Pre K3 and Pre M3) were not in use. Only one chlorinated sample, M3, contained sufficiently high bacterial counts to permit a clear assessment of the effect of temperature. Plates incubated at 30°C yielded a mean of 3.0 CFU per ml whereas those incubated at 20°C gave a mean of 300 CFU per ml. Temperature shifted plates showed an intermediate level of recovery of 100 CFU per ml. These data are consistent with the findings for source water, CPR1, in which the highest counts were also found on plates incubated at 20°C. Similar results were recorded for other chlorinated samples, but because of the high levels of residual chlorine, counts were quite low. One other noteworthy sample was from Lincoln Reservoir (I3) in which 43 CFU per ml were recorded from an everage of three DP plates at 20°C, whereas only 18 per ml were found at 30°C. The same trend was noted for samples from this site plated on CPS medium. When compared to 30°C counts, the 20°C to 30°C shift produced higher plate counts for CPR1, L3 and M3 samples and lower plate counts for I3 and K3. Myrtle plate counts were the same for 20°C→30°C and 30°C. An incubation temperature of 11°C was tested for samples from Pre I3, I3, Pre L3 and L3 to determine if a lower incubation temperature would increase recoveries when compared to 20°. Increased recoveries were found for two of the reservoirs, Pre I3 and Pre L3, whereas chlorinated samples did not show statistically significant differences due to the low counts.

Thus, it appears that, for bacteria indigenous to the Seattle water distribution system, use of an incubation temperature of 30°C results in reduced bacterial plate counts. Increased bacterial counts could be obtained by shifting plates initially incubated at 20°C to 30°C, but some bacteria

were unable to grow at 30°C even following this shift indicating their psychrophilic behavior, at least on initial isolation. It may be that the highest counts during winter surveys would be obtained at incubation temperatures below 20°C.

Effect of physical and chemical factors of treatment system on recovery of viable bacteria from treated samples. Statistical analyses were performed to determine whether any of the measured physical and chemical factors of the treatment and distribution processes produced a reproducible effect on bacterial recovery from chlorinated samples. Thus, residual chlorine concentration, temperature, and pH were included in a multiple linear repression analysis with CFU on DP and CPS media. No significant correlations, either positive or negative, were observed. The number of CFU on DP and CPS from chlorinated samples were compared to the number of CFU on the same respective media recovered from the immediately preceding non-chlorinated water (i.e., reservoir sample), expressed as a percent surviving chlorination, and normalized using arc sin transformation. These normalized percents were also compared to chlorine concentrations, temperature, and pH using multiple linear regression analysis, but again no significant correlations, either positive or negative, were found.

The number of CFU on DP and CPS from chlorinated waters and the normalized percentages were also used in multiple regression analyses versus the following combinations: chlorine + temperature + pH; chlorine + temperature; chlorine + pH; and temperature + pH. No significant relationships were found.

Direct acridine orange microscopic counts. Acridine orange (AO) counts were affected by chlorination. For example, on September 2, 1981 (Table 14), an approximate 50% reduction in cell numbers was noted following chlorination (CPTO) of the source water (CPR1). A somewhat greater percentage reduction was noted following chlorination (PL2) of Lake Youngs (LY). Smaller reductions were not noted following chlorination in the in-city reservoirs, although reductions of up to 50% were sometimes found (e.g., M3). Similar results were noted on November 12, 1981 (Table 15). A high reduction was noted in acridine orange counts following chlorination of the source water (about 40% at CPTO) and smaller reductions were noted after the in-city reservoirs. Indeed, most of the final chlorinated samples in this survey showed only a minor reduction in numbers compared to their reservoirs or none at all (e.g., K3).

Viable plate counts from all samples were lower than the acridine orange counts. This is to be expected inasmuch as the microscopic count would include both viable and non-viable bacteria. The source water showed recoveries that are typical of oligotrophic alpine lakes (Staley et al., 1982), i.e., about 1% of the total count was cultivable on DP medium. As expected, chlorinated samples usually showed even lower recoveries. Frequently, the ratio of viable to AO counts in Lake Youngs and some of the in-city reservoirs exceeded 10% (high values ranged from 11.8% to 42.4%). Such high recoveries suggest that the water in Lake Youngs and many of the in-city reservoirs is eutrophic, whereas the source water is oligotrophic to mesotrophic. Poor recoveries were obtained for only one reservoir, I3, on

TABLE 14. COMPARISON OF ACRIDINE ORANGE (AO) DIRECT MICROSCOPIC COUNT WITH VIABLE COUNT.[1] CEDAR RIVER WATERSHED. SEPTEMBER 2, 1981.

						- Percent Viable -	
Location	AO count	Std. error	DP	Std. error	SPC	DP/AOx100	SPC/AOx100
CPR1	8.6×10^5	2.8×10^4	3.6×10^3	- (1)[2]	-	0.42	-
CPTO	4.2×10^5	2.7×10^4	1.2×10^4	- (1)	-	2.86	-
LY	8.5×10^5	5.7×10^4	1.0×10^5	2.4×10^4	-	11.8	-
CPL2	8.1×10^5	2.1×10^4	6.0×10^1	- (1)	-	0.019	-
Reservoir L3	5.3×10^5	1.8×10^4	1.6×10^5	2.4×10^4	7.4×10^1 (1)	30.2	0.014
L3	3.5×10^5	1.3×10^4	3.1×10^3	2.0×10^2	-	0.89	-
Reservoir M3	1.2×10^6	1.7×10^4	4.0×10^5	3.6×10^4 (2)	6.4×10^1 (1)	33.6	0.0054
M3	5.9×10^5	1.4×10^4	1.6×10^5	9.2×10^3 (2)	$<1.0 \times 10^0$ (1)	27.1	<0.00017
Reservoir I3	2.8×10^5	5.9×10^4	1.1×10^3	1.1×10^2	5.8×10^1 (1)	0.39	0.021
I3	2.2×10^5	1.9×10^4	5.1×10^2	9.3×10^1	$<1.0 \times 10^0$ (1)	0.23	<0.00046
Reservoir K3	2.1×10^5	4.0×10^4	8.9×10^4	1.2×10^4	1.0×10^2 (1)	42.4	0.048
K3	1.8×10^5	2.5×10^4	1.7×10^3	2.6×10^2	$<1.0 \times 10^0$ (1)	0.94	<0.00056

[1] Number of bacteria per ml. DP - dilute peptone medium; SPC - standard plate count medium.

[2] Normally three plates were used for each viable enumeration; if fewer than three plates were available for final count (due to overgrowth or other problems) this is indicated by parenthetical number.

TABLE 15. COMPARISON OF ACRIDINE ORANGE (AO) DIRECT MICROSCOPIC COUNT WITH VIABLE COUNT[1]
CEDAR RIVER WATERSHED NOVEMBER 12, 1981

Location	Residual Chlorine	AO count	Std. error	DP	Std. error	SPC	Std. error	- Percent Viable - DP/AOx100	SPC/AOx100
CPR1	--[2]	$1.3x10^6$	$1.4x10^4$	$1.4x10^4$	$1.1x10^3$	$2.6x10^1$	$1.2x10^1$	1.08	0.0020
CPTO	--	$7.6x10^5$	$3.6x10^4$	$9.8x10^1$	- (1)[3]	$3.0x10^0$	$3.0x10^0$	0.013	0.00049
LY	--	-		-		-		-	-
CPL2	--	$6.0x10^5$	$3.5x10^4$	$1.9x10^1$	$7.2x10^0$ (2)	$0.3x10^0$	$0.6x10^0$	0.0032	0.000050
Reservoir L3	--	$9.1x10^5$	$4.8x10^4$	$<0.3x10^3$	-	$0.3x10^0$	$0.6x10^0$	<0.033	0.000033
L3	0.60	$8.2x10^5$	$4.3x10^4$	$<0.3x10^1$	-	$<0.3x10^0$	-	<0.0034	<0.000037
Reservoir M3	--	$1.2x10^6$	$7.9x10^4$	$4.6x10^5$	$2.1x10^4$	$2.3x10^0$	$2.5x10^0$	38.3	0.00020
M3	0.62	$1.1x10^6$	$7.4x10^4$	$1.6x10^5$	$1.1x10^4$	$1.3x10^0$	$1.2x10^0$	14.5	0.00012
Reservoir I3	--	$1.2x10^6$	$5.3x10^4$	$1.5x10^2$	$5.2x10^1$	$<0.3x10^0$	-	0.041	<0.000025
I3	0.75	$9.6x10^5$	$5.7x10^4$	$5.0x10^0$	$1.4x10^0$ (2)	$2.0x10^0$	$1.8x10^0$	0.00052	0.00021
Reservoir K3	--	$1.3x10^6$	$1.8x10^5$	$3.4x10^3$	$1.8x10^3$	$1.3x10^0$	$1.6x10^0$	0.26	0.00010
K3	0.50	$1.3x10^6$	$9.1x10^4$	$7.0x10^1$	$4.3x10^1$ (2)	$<0.3x10^0$	-	0.0054	<0.000023

1 Number of bacteria per ml. DP - dilute peptone medium; SPC - standard plate count agar.

2 Not determined for chlorine concentration; value is expected to be 0.

3 Normally three replicate plates were prepared for each enumeration. If fewer than three plates were readable (because of overgrowth, etc.) then number of plates is recorded parenthetically.

September 2. In contrast, only one reservoir, M3, showed high recoveries on November 12.

Diversity Indices. Visual examination of plates from the September 2, 1981 survey indicated there was a dramatic change following chlorination of the source water. Whereas most of the colonies found in the drinking water samples were pigmented and consisted of relatively few colony types, the source water (i.e., CPR1) contained many nonpigmented types and a greater variety of colony types. These observations suggested that there was a marked change in the diversity of bacteria following chlorination. To quantitate the diversity values of the source water and the distribution water, the Shannon-Weaver index was used (cf. Sites, Materials, and Procedures section). Table 16 shows the results for September 2, 1981.

The diversity index of the source water was 4.14. This compares favorably to a value of 4.58 calculated for source water in a previous survey (Fiksdal et al., 1982). Diversity values for the chlorinated samples were 1.85 for I3, 2.81 for K3, 1.23 for L3, and 1.08 for M3. These were all based upon a count of 55 colonies, except for M3 where only 40 colonies were available. The slow growing Hyphomonas colonies were not detectable at the time of the 28 day count and were thus not included in the initial diversity index calculations. Therefore the diversity index values for those samples that contained Hyphomonas colonies resulted in an increase in diversity in all three instances where they were detected.

It is apparent that in the chlorinated samples collected on September 2, 1981, the diversity indices were substantially lower than in the source water. This result is entirely consistent with the data collected in earlier surveys where diversity index values have been calculated (Fiksdal et al., 1982). Thus, we conclude that the disinfection process and distribution in this system commonly and perhaps always, results in a selection of relatively few bacterial species in comparison with the source water.

TABLE 16. DIVERSITY INDICES OF SOURCE WATER (CPR1) AND CHLORINATED SAMPLES COLLECTED SEPTEMBER 2, 1981, AND COUNTED AFTER 28 AND 40 DAYS

Sample	28 days No. bacteria/ml	D.I.[1]	40 days No. bacteria/ml[3]	D.I.[2]
CPR1	3,600	4.14	-	-
I3	210	1.85	510	2.45
K3	1,700	2.81	-	-
L3	2,900	1.23	3,070	1.45
M3	150,000	1.08	160,000	1.55

[1] Diversity index based upon 55 colonies except for M3 in which 40 colonies were used.

[2] Diversity index based upon Hyphomonas colonies. Total colonies counted for I3 were 81; L3 were 58; and M3 were 44.

[3] 40 day counts were determined from 28 day plates that had been refrigerated. Increased counts on plates were due to growth of Hyphomonas.

SECTION 5

CHARACTERIZATION AND IDENTIFICATION OF BACTERIA FROM CHLORINATED DRINKING WATERS

Introduction

The major purpose of this part of the investigation was to identify the predominant heterotrophic bacteria from two different drinking water systems. The process of identification implicitly assumes that the organisms that are being investigated are known organisms that have been previously characterized, described, and named. When this investigation was proposed, it was anticipated that most, if not all, isolates to be found in the chlorinated drinking waters would be strains of known organisms. If so, then it would have been a straightforward process of characterizing isolates and comparing them to known organisms. The results indicated, however, that this was not true. Many of the organisms were not identifiable to known species. Furthermore, because of their slow growth and general non-reactivity, they posed special problems for characterization. Nonetheless, tests were developed that permit a phenotypic characterization and description of these bacteria, and a dichotomous flow-chart for the characterization process is included with this report.

Materials and Methods

Selection of isolates. Because there is considerable variation in water chlorination systems in the U.S. and it was considered desirable to obtain isolates from different sources, strains were selected from two completely different water treatment systems. Half of the collection (about 50 strains) were obtained from the Seattle water distribution system which is described earlier in this report (Section III A). Of these, half were collected during the summer and the remainder were collected during the winter.

Seattle summer strains were isolated from the September 2, 1981 (Table 11) samples. In this particular survey colony types were identified by morphology on the dilute peptone medium used in determination of diversity index values (Table 16). Then representatives of predominant colony types were selected from several of the chlorinated samples. Thus, the numbers per ml can be determined for each of these colony types. Strain numbers and numbers per ml are provided in Table 17. Although more than 25 strains were initially isolated, only 23 remained cultivatable sufficiently long for characterization.

Table 17. BACTERIAL STRAINS SELECTED FROM CHLORINATED DRINKING WATERS IN SEATTLE, SUMMER ISOLATES, SEPTEMBER 2, 1981

Source[1]	Strain No.	Colony Type	DP Isolation plate	Number per ml
I3	I3 A1	A	Pink, convex, entire	100
	I3 C1	C	White convex, entire	15
	I3 D1	D	Yellow-orange center, umbonate, entire	3.8
	I3 E1	E	Cream, convex, entire[3]	3.8
	I3 E2	E	Cream, convex, entire[3]	(3.8)[2]
	I3 F1	F	Yellow-orange, convex entire	50
	I3 K1	K	Pink, convex, small	300
			Total	473
			Total viable count of sample	510
K3	K3 A1	A	Yellow-orange, umbonate, entire	190
	K3 A2	A	Yellow-orange, umbonate, entire	(190)
	K3 B1	B	Yellow-orange, convex, entire	660
	K3 B2	B	Yellow-orange, convex, entire	(660)
	K3 C1	C	Grainy, colorless, convex, entire	190
	K3 D1	D	Pink, convex, entire	63
	K3 E1	E	Cream, pulvinate, undulate[3]	63
	K3 F1	F	Clear white, pulvinate, concave, undulate	31
	K3 G1	G	Yellow, convex, entire	31
			Total	1,228
			Total viable count of sample	1,700
L3	L3 A1	A	Yellow	2,100
	L3 A2	A	Yellow	2,100
	L3 B1	B	Deep red, convex, entire	510
	L3 B2	B	Deep red, convex, entire	(510)
	L3 C1	C	White, convex, entire[3]	510
	L3 C2	C	White, convex, entire[3]	(510)
			Total	1,020
			Total viable bacteria in sample	3,100
M3	M3 A1	A	Yellow-orange, convex, entire	100,000
	M3 A2	A	Yellow-orange, convex, entire	(100,000)
	M3 B1	B	Light brown, convex, entire	17,000
	M3 B2	B	Light brown, convex, entire	(17,000)
	M3 C1	C	White, convex, entire	3,300
	M3 D1	D	Yellow, convex, entire	3,300
	M3 D2	D	Yellow, convex, entire	(3,300)
			Total	123,600
			Total viable bacteria in sample	150,000

1 Final chlorinated sample (see Table 5)
2 Numbers in parentheses are not included in total count bacause the colony type has already been previously included.
3 On cultivation on richer media these become pigmented yellow to orange-brown.

Isolates were also obtained in the winter months. Because such high chlorine residuals were present at these times, too few isolates were obtained in either the November 12 survey (Table 12) or February 24 (Table 13) survey for a complete collection of 25 from one sampling date. Also, for this reason, it was not possible to determine diversity indices. However, by combining isolates from the November 12 and February 24 surveys a collection of 28 strains was obtained (Table 18).

The other half of the collection (about 50 strains) was obtained from the Cincinnati water treatment processes and distribution system. The Cincinnati treatment facility consists of flocculation, coagulation, sedimentation, filtration and culminates with disinfection by chlorination. Approximately half of the Cincinnati collection (25 strains) was obtained during the summer months (Table 19) whereas the remaining half was collected during the winter months (Table 20). Some of the strains were collected from chlorinated filter bed influent and effluent, others from the distribution mains, and the remainder from dead end lines of the distribution mains. These isolates were randomly selected from the R2A isolation plates.

Identification Methods

Gram Stain:

Cells were obtained from a young culture of CPS-CAA broth or R3A (for media composition see Table 21) slant and allowed to dry on a glass slide before heat fixing. The smear was flooded with crystal violet solution for 1 min, washed with iodine solution and flooded with iodine solution for 1 min. It was then washed with tap water and decolorized (50:50 ethanol:acetone) until solvent flowed colorlessly from slide, then immediately washed with tap water and blotted dry. Smears were then counter-stained with safranin. Gram positive organisms stained purple, Gram negative organisms stained red. Gram variable cells included both Gram positive and negative cells. One positive, one negative, and one uninoculated control were included on a slide along with as many as five test strains (Lennette, C.H. et al., eds. "Manual of Clinical Microbiology", third edition). Controls used were Staphylococcus aureus, Escherichia coli, and Cellulomonas biozotea.

Cellular Morphology:

Wet mounts were examined of young cultures grown in broths or slants of CPS-CAA, DP, MMB, and Caulobacter medium. A Zeiss Ultraphot phase contrast microscope was used.

Colonial Morphology:

Initial colony morphology was determined from DP isolation plates on Seattle isolates. Subsequently a loop of cell suspension was streaked for isolation on CPS-CAA plates and incubated at 20°C. Morphological description was taken from an isolated representative colony. Pigmentation was also determined from this plate.

TABLE 18. BACTERIAL STRAINS SELECTED FROM CHLORINATED DRINKING WATERS IN SEATTLE, WINTER ISOLATES

Source[1]	Date of Collection	Strain No.	Colony Type (DP isolation plate)
I3	February 24, 1982	I3 2/24 A1	Pink center, flat, semi-opaque
	"	I3 2/24 A2	Pink center, flat, semi-opaque
	"	I3 2/24 B1	Yellow, flat, semi-opaque, smooth
	"	I3 2/24 C1	White, dull, semi-opaque
	"	I3 2/24 D1	Light yellow, large mucoid
	"	I3 2/24 E1	Dark yellow, opaque
	"	I3 2/24 F1	White with outer ring
	"	I3 2/24 G1	Tan, wrinkled center
	"	I3 2/24 H1	White, raised
K3	November 12, 1981	K3 11/12 A1	Yellow, irregular edge
	"	K3 11/12 A3	Yellow, irregular edge
	"	K3 11/12 B1	Light yellow, flat
	"	K3 11/12 C1	Light yellow, pulvinate
	"	K3 11/12 D1	Yellow-orange, dark center
L3	February 24, 1982	L3 2/24 A1	White, flat opaque
	"	L3 2/24 B1	Yellow, smooth
	"	L3 2/24 C1	Light yellow, shiny
	"	L3 2/24 D1	Yellow, pulvinate
	"	L3 2/24 F1	White, opaque
	"	L3 2/24 G1	Yellow
M3	November 12, 1981	M3 11/12 A1	Tan, mucoid
	"	M3 11/12 B1	Tan, flat, mucoid
	"	M3 11/12 B2	Tan, flat, mucoid
	"	M3 11/12 B3	Tan, flat, mucoid
	"	M3 11/12 C1	Purple-violet
Myrtle	February 24, 1982	Myr 2/24 A1	Dark brown
	"	Myr 2/24 B1	Yellow, mucoid
	"	Myr 2/24 C1	Yellow

1 Source of final chlorinated sample from Table 5.

TABLE 19. BACTERIAL STRAINS SELECTED FROM CHLORINATED DRINKING WATER IN CINCINNATI. SUMMER ISOLATES, JULY 7, 1982

Source	Strain number	Colony type on R3A medium
All strains collected from a flushed dead-end fire hydrant about 100 meters from point where strains 6-1 through 6-16 were collected (see Table 20)	707-1	yellow - fried egg with subunits present
	707-2	bright orange - entire
	707-3	white - entire - variable colony size
	707-4	cream - raised center - entire
	707-5	large cream mucoid - entire
	707-6	small white - entire
	707-7	cream - entire - variable size (like 707-3)
	707-8	bright orange - entire (similar to 707-2)
	707-9a	white - entire
	707-9b	large white-cream - entire - with darker colony segments
	707-10	small cream - variable size - entire[1]
	707-11a	small white - entire[1]
	707-12	large mucoid cream - entire
	707-14	large mucoid cream - entire
	707-15	white-cream - mucoid
	707-17	large orange - entire
	707-18	light yellow - entire
	707-20	large white-cream with darker colony segments
	707-21	yellow - most entire but some have singular edge
	707-23	large raised center white - color variation
	707-24	large orange - entire
	707-25	white medium - entire
	707-26	large cream-white - color variation (like 707-9b)
	707-27	small yellow entire edge
	707-28	cream-yellow - entire

1 On prolonged cultivation these become light yellow in pigmentation.

TABLE 20. BACTERIAL STRAINS SELECTED FROM CHLORINATED DRINKING WATER IN CINCINNATI, WINTER ISOLATES

Source	Strain number	Colony type on R3A plates.
Not recorded	2	Yellow, translucent, entire
"	4	Yellow, translucent, pulvinate
"	5	Yellow, translucent edge, opaque centers, entire
"	8	Yellow, translucent edge, semi-opaque center, entire
"	10	Orange-brown, translucent, entire
"	14	Pink, translucent edge, semi-opaque center, entire
"	16	Light yellow, semi-opaque, entire
"	21	Pink, translucent edge, semi-opaque center, entire
"	35	Orange-brown, translucent, entire
"	60	White, flat, opaque, entire
"	62	Yellow, opaque, entire
Chlorinated influent to filter bed	4-3	Orange-brown, smooth, pulvinate
	4-4	Yellow, translucent, semi-opaque center, entire
Filter-bed effluent	5-5	Orange-brown, smooth pulvinate
Distribution mains at ends of distribution system	6-1	Pink, translucent edge, opaque center, entire
	6-2	Yellow, translucent, entire
"	6-5	Orange-brown, semi-opaque, pulvinate
"	6-7	Dull white, opaque, pulvinate
"	6-8	Shiny white, opaque, mucoid
"	6-9	Cream, translucent, flat, entire
"	6-14	Cream, translucent, umbonate, entire
"	6-15	White, translucent, semi-opaque center, convoluted edge
"	6-16	Yellow, translucent, mucoid, entire

TABLE 21. STANDARD GROWTH AND MAINTENANCE MEDIA[1]

CPS Agar

	Amount per l
Sodium Caseinate	0.5 g
Peptone	0.5 g
Soluble starch	0.5 g
Glycerol	1.0 ml
K_2HPO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.05 g
$FeCl_3$	4 drops of 0.01% solution
Agar	15.0 g

pH 7.0-7.4

CPS-CAA Agar

Casamino acids is substituted for caseinate and starch in CPS medium at a concentration of 0.2 g/l.

DP Agar

	Amount per l
Bacto-Peptone	0.1 g
Hutners salts solution (modified)[2]	20.0 ml
Vitamin solution[2]	10.0 ml
Agar	15.0 g

pH 7.0

R2A Agar (R3A is 2xR2A)

	Amount per l
Yeast extract	0.5
Proteose peptone No. 3	0.5
Casamino acids	0.5
Glucose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
K_2HPO_4	0.3
$MgSO_4 \cdot 7H_2O$	0.05
Agar	15.0

pH 7.2

1 All media made to volume with distilled water.

2 Staley, 1982.

Anaerobic Growth:

A loopful of cell suspension was used to streak a single line onto media in regular size petri plates (100 x 15 mm). As many as 10 organisms were tested on one plate. One set of plates was placed in an anaerobic jar (BBL Gas Pak). Prior to streaking, holes were made in the lids of these plates using a hot fork so that anaerobiosis could be attained quickly. The other set was incubated aerobically as a control. Media tested included CPS-CAA, MMB, and Caulobacter medium supplemented with glucose (0.2%). Controls included Escherichia coli, Pseudomonas aeruginosa and Acinetobacter calcoaceticus.

Oxidase test:

A 1% solution of N, N, N^1, N^1,-tetramethyl-p-phenylene-diamine-2HCl (22 to 24 drops) was used to moisten Whatman No. 3 qualitative filter paper ($\sim$ 8.5 cm diameter) placed in petri dishes (100 x 15 mm). The paper was allowed to dry at room temperature or in the refrigerator and was used immediately or placed in the refrigerator. Cell paste from a young culture grown on a carbohydrate free medium (nutrient agar, dilute peptone, or Caulobacter medium) was smeared on the impregnated paper with a platinum loop. Cells turning purple within 10 s constituted a positive reaction. Most reactions were immediate. Controls were done on each pad prior to testing and if there were questionable results, new pads were used. Impregnated pads were stored in the dark in the refrigerator (Lennette, E.H. et al., eds. "Manual of Clinical Microbiology", third edition; personal communication, Elliot Juni). Controls: Escherichia coli, Pseudomonas aeruginosa.

Growth on mineral media with carbon sources:

The ability to grow in a mineral medium with and without 0.2% mannitol, glutamate and acetate as the sole carbon source and $(NH_4)_2SO_4$ as the nitrogen source was tested by inoculating a broth of the medium with a loopful of cell suspension. Turbidity was measured visually by comparison with uninoculated controls. Two media were used including MMB (Staley, 1982) and the medium of Stanier et al. (1966) supplemented with a vitamin solution (Staley, 1968). Controls included Pseudomonas aeruginosa and Cellulomonas biozotea.

O/F, oxidation/fermentation:

Initial attempts to utilize the Hugh Leifson O/F medium (Hugh and Leifson, 1953) were disappointing; only 11 out of 48 of the Cincinnati and Seattle strains tested initially grew. Thus, the O/F medium for non-fermenting Gram negative bacilli (Lennette, E.H. et al., eds. "Manual of Clinical Microbiology", 3rd edition) supplemented with Hutner's salts (Staley, 1982) and vitamins (Staley, 1982) was also employed. Tubes were inoculated by loop at room temperature using a cell suspension. One stab was made into the bottom of each tube. The fermentative tubes were overlaid with sterile vaspar (50:50 petroleum jelly and paraffin) or mineral oil. A final 1.0% carbohydrate concentration (glucose) and basal medium control tubes were

used. Tubes were assessed for both growth and pH change (acid, alkaline, or no change). Uninoculated control tubes were used to ascertain a change in pH when comparing test organisms. A set of tubes was also tested by steaming the tubes to drive off the oxygen, quickly chilled in an ice bucket and inoculated. Controls used were Escherichia coli and Pseudomonas aeruginosa.

PHB:

A loop of cell suspension was used to inoculate a mineral medium with β-hydroxybutyrate (0.5%) as the carbon source. If growth occurred the cells were observed by phase microscopy and the presence of inclusions was noted. Organisms from tubes showing growth were examined at regular intervals before concluding a negative reading (Barre and Henriksen, 1976). Presumptive inclusions were verified by staining with Sudan Black B. Controls used were Moraxella osloensis and Escherichia coli.

Spore formation:

A loop of cell suspension was used to inoculate 5 ml of CPS medium. This medium was used because (a) it is used routinely in the subculturing of the organisms, (b) the starch and caseinate are regarded to favor spore formation in some bacteria. When the culture to be tested was no longer in exponential growth stage, a loopful was streaked from the broth tube onto a half section of a CPS or CPS-CAA agar petri dish. A bath was heated to 80°C and the culture tubes immersed. A thermometer was placed inside an uninoculated control tube in order to determine temperature. Tubes were held for 10 min at 80°C ± 0.5%C. Tubes were then removed and placed in an ice bath. A loopful from each tube was streaked onto the uninoculated adjoining section of the CPS or CPS-CAA plate onto which the positive controls were previously half streaked. The plates were placed in the 20°C incubator and both sides were examined for growth after incubation (Gerhardt et al., eds. 1981). Controls used included Bacillus species and Escherichia coli.

Growth at pH 4.5 and acid production from ethanol (test for acetic acid bacteria):

Growth at pH 4.5 was tested by adjusting CPS-CAA broth to pH 4.5. A loop of cell suspension was used to inoculate the broth. Growth was compared to a control broth at neutral pH (7.0) (Bergey's Manual of Determinative Bacteriology: 8th edition, p. 251). Organisms were also tested for growth and acid production on Carr's ethanol medium (Carr, 1960). A loop of a cell suspension was used to inoculate a slant. A control medium with no carbon source was also inoculated. Controls used were Acetobacter aceti subsp. aceti, Gluconobacter oxydans subsp. oxydans and Cellulomonas biozotea.

Nitrification:

A loopful of cell suspension was used to inoculate Winogradsky's modified medium to test for the oxidation of $(NH_4)_2SO_4$ to nitrite. The presence

of nitrite was tested for and, if present, constitutes a positive test (Dutka, 1978).

Cellulose Degrading Ability:

CPS-CAA broth with cellulose powder added (Whatman, 0.2 g/l). Tubes were inoculated with a loop of cell suspension. Degradation and clearing of the cellulose was looked for as a positive test. Cellulolytic activity was also tested with trypticase soy broth containing a strip of cellulose filter paper (Whatman 1 qualitative) added to it (James E. Gannon, personal communication). Controls used were Cellulomonas biozotea, Cellulomonas fimi, and Escherichia coli.

Growth Media Comparison:

Loops of cell suspensions were used to streak slants of dilute peptone agar (DP), caseinate-peptone-starch (CPS), and caseinate peptone starch-casamino acids (CPS-CAA, Table 21). Growth was compared on each of the respective media.

Pigmentation/Fluorescence:

A loop of cell suspension was streaked on a plate or slant of King A or King B medium. Growth, pigmentation, and fluorescence was recorded. Fluorescence was evaluated by illuminating the plate with an ultraviolet lamp (King et al., 1954). Controls used included Pseudomonas fluorescens, Pseudomonas aeruginosa, and Escherichia coli.

Growth Temperature:

A loopful of cell suspension of each organism was streaked on four slants of CPS-CAA agar. A set of slants was incubated aerobically for 25-35 days at each of the following temperatures: 11°, 30°, and 37°C with the control set incubated at 20°C. Results were recorded as negative, +, ++, or +++ with + indicating light growth and +++ indicating heavy growth.

Cell Suspensions:

A heavy loop inoculum was streaked on a CPS-CAA agar slant and incubated at 20°C overnight. The cells were then washed off with approximately 0.5 ml of sterile peptone buffer (0.1% bacto-peptone). The cells were placed in sterile Eppendorf tubes, vortexed, and centrifuged. The cells were resuspended in 1 ml of sterile physiological saline, pipetted into sterile screw capped tubes, and placed on ice while being held for inoculation.

Incubation:

All media were incubated at 20°C unless otherwise specified. The tests were incubated 25 or 35 days before the final readings were recorded.

Special Media Specifications:

Unless otherwise stated all broths used in culturing the organisms contained 0.1% agar. pH was adjusted before autoclaving.

Maintenance, storage, and preservation of cultures:

Strains were transferred onto a variety of media for maintenance and storage. R2A and R3A were commonly used for the Cincinnati strains whereas CPS, CPS-CAA, MMB or DP were used for the Seattle isolates. Stocks were routinely maintained in the refrigerator. However, longer term preservation was effected once strains were purified by freezing them in glycerol. For glycerol freezing cells were harvested from stationary phase slants with dilute peptone diluent or taken directly from a broth. Three ml of the suspension was added to a 1 dram vial previously autoclaved with 0.53 ml of glycerol. Two sets of vials were frozen, one at -22°C and another at -80°C. A plate was streaked prior to freezing to verify purity and viability and tests of viability were made two to four weeks after freezing. The procedure was adapted from Hollander and Nell (1954).

Comparison strains:

A number of comparison strains were obtained from other investigators and the American Type Culture Collection to permit a direct comparison for phenotypic testing. The strains and their sources are shown in Table 22.

Quality control:

Quality control procedures were used to test media, reactions of organisms, quality of distilled water, and confirmation of results.

Results

Morphology. Each strain was examined with the phase contrast microscope using wet mount preparations. Cell shape was noted and recorded. Cells were also examined for motility. Gram stains were made (Tables 23-26).

Most of the organisms were Gram-negative rods. However, some of the strains were morphologically distinctive. The most morphologically distinctive strains were prosthecate bacteria. Some of these organism can be identified to genus on the basis of their morphological features alone. For example, the genus Caulobacter contains Gram-negative rods that have polar prosthecae (stalks) and are motile. Strains of Caulobacter were noted both from the Seattle distribution system as well as the Cincinnati distribution system. Members of this genus have been known to be aquatic bacteria since the time of Henrici and Johnson (1935) and were even reported in the Chicago water supply much earlier [(Jones, 1905); however, Jones thought the polar stalk was a flagellum and therefore did not assign it to a new genus]. To our knowledge however, caulobacters have not previously been demonstrated in chlorinated tap water. Of the 102 strains examined 9 were of the genus Caulobacter. This includes eight from the Seattle drinking water system and one from the Cincinnati drinking water distribution system (707-20). This genus was found in both the summer and winter surveys in Seattle. In the

TABLE 22. COMPARISON STRAINS USED IN INVESTIGATION

Species	Source
Acetobacter aceti subsp. *aceti*	ATCC 15973
Aeromonas hydrophila	Scott Rippey (V.J. Cabelli's laboratory)
Arthrobacter globiformis	ATCC 8010
Cellulomonas biozotea	ATCC 486
Cellulomonas fimi	ATCC 484
Flavobacterium aquatile	ATCC 11947
Flavobacterium capsulatum	ATCC 14660
Flavobacterium meningosepticum	U.W. Hospital
Gluconobacter oxydans subs. *oxydans*	ATCC 19357
Gluconobacter oxydans subs. *melanogenes*	ATCC 33447
Pseudomonas diminuta	1958 B (Berkeley 501)
Pseudomonas diminuta	ATCC 11568
Pseudomonas fluorescens	U.W. Hospital
Pseudomonas maltophilia	ATCC 17450
Pseudomonas maltophilia	ATCC 17666
Pseudomonas maltophilia	U.W. Hospital
Staphylococcus aureus	U.W. Hospital
Vibrio parahaemolyticus	U.W. Hospital
Xanthomonas campestris	ATCC 9563
Xanthomonas campestris	NCPP 13528

TABLE 23. CELL MORPHOLOGY AND TENTATIVE GENERIC ASSIGNMENT OF SEATTLE SUMMER ISOLATES FROM DRINKING WATER

Strain Number	Gram Stain	Motility[1]	Cell Morphology (CPS or DP slant)[2]
I3-A1	-	+	straight or bent large rods with granules
I3-C1	+	-	small rods, forms chains
I3-D1	+	-	*Micrococcus* sp.
I3-E1	-	-	medium rods
I3-E2	-	-	medium to long rods
I3-F1	-	-	small rods, some chains (non-motile?)
I3-K1	-	-	*Hyphomonas* sp.
K3-A1	-	+	*Caulobacter* sp.
K3-A2	-	-	*Caulobacter* sp.
K3-B1	-	+	slightly bent rod
K3-B2	-	-	short to long rod
K3-C1	-	-	short, bent rods
K3-D1	-	-	medium rods
K3-E1	-	-	medium to long rod with inclusions
K3-F1	-	-	small rod, rosettes
K3-G1	-	-	long rods
L3-A1	+	-	medium rods
L3-A2	+	-	medium rods
L3-B1	-	-	large rods
L3-B2	-	-	large rods
L3-C2	-	-	medium rods
M3-A1	-	-	long rods, *Caulobacter* sp.
M3-A2	-	-	long rods, *Caulobacter* sp.
M3-B1	-	+	*Caulobacter* sp.
M3-B2	-	-	medium rods; large swollen forms, *Caulobacter* sp.
M3-C1	-	+	small rods, *Pseudomonas* sp.
M3-D1	-	-	large rods, rosettes, *Caulobacter*
M3-D2	-	-	large rods, rosettes, *Caulobacter*

1 If +, motility was observed in wet mount; - indicates that motility was not observed (it is possible that - strains would be motile or have motile stages that were not deteced under conditions tested).

2 Cell diameter for typical strains ranged from 0.4 - 1.0 µm. Thin rods would be less than 0.4 µm and fat rods would be greater than 1.0 µm in diameter.

TABLE 24. CELL MORPHOLOGY OR TENTATIVE GENERIC ASSIGNMENT OF SEATTLE ISOLATES FROM DRINKING WATER

Strain Number	Gram Stain	Motility[1]	Cell Morphology (CPS or DP slant)[2]
I3 2/24 A1	-	-	short, fat rods with inclusions
I3 2/24 A2	-	-	as above
I3 2/24 B1	-	-	medium rods, attached at one pole, some elongated cells
I3 2/24 C1	-	-	small rods
I3 2/24 D1	-	+	*Caulobacter* sp.
I3 2/24 E1	-	-	rods of irregular length, spindle forms
I3 2/24 F1	-	+	medium rod, digests agar (DP)
I3 2/24 G1	+	+	coccus
I3 2/24 H1	+	+	various width rods, spindle shaped
K3 11/12 A1	-	-	medium rods, form rosettes (no stalk observed)
K3 11/12 A3	+	-	cocci in packets, *Staphyloccus*
K3 11/12 B1	-	-	short rods
K3 11/12 C1	-	-	medium to long rods
K3 11/12 D1	-	-	short rods
L3 2/24 A1	+	-	coccus, *Staphylococcus* sp.
L3 2/24 B1	-	-	medium rods, rosettes
L3 2/24 C1	+	-	coccus, some pairs, *Staphylococcus* sp.
L3 2/24 D1	-	+	*Caulobacter* sp.
L3 2/24 F1	+	-	coccus, *Micrococcus* sp.
L3 2/24 G1	-	-	thin rods of medium length
M3 11/12 A1	-	-	medium rods, some cells elongated
M3 11/12 B1	-	-	medium rods
M3 11/12 B2	-	-	medium rods
M3 11/12 B3	-	-	medium rods, some chinese characters
M3 11/12 C1	-	-	short rods, in pairs, pointed ends
Myt 2/24 A1	-	-	short rods, rosettes
Myt 2/24 B1	-	-	medium rods
Myt 2/24 C1	-	-	medium rods

1 As in Table 23.
2 Cell diameter for typical strains ranged from 0.4 - 1.0 μm. Thin rods would be less than 0.4 μm and fat rods would be greater than 1.0 μm in diameter.

TABLE 25. CELL MORPHOLOGY AND TENTATIVE GENERIC ASSIGNMENT OF CINCINNATI SUMMER ISOLATES FROM DRINKING WATER

Strain Number	Gram stain	Motility	Cell morphology (R3A broth) [1]
707-1	-	-	very thin rods
707-2	-	-	long rods, some filaments
707-3	-	-	short, fat rods
707-4	-	-	no growth
707-5	-	+	fat rods, swollen forms
707-6	-	-	similar to 707-5
707-7	-	-	short rods, like number 707-3
707-8	-	-	long rods, like 707-2
707-9a	-	+	identical to 707-5
707-9b	-	+	identical to 707-5
707-10	-	-	short rods, chins
707-11a	-	-	similar to 707-10
707-12	-	+	thin rods
707-14	-	-	large rods, swollen forms
707-15	-	-	large rods, rosettes
707-17	-	-	similar to 707-2
707-18	-	-	similar to 707-15
707-20	-	+	*Caulobacter* sp.
707-21	variable	-	medium rods
707-23	-	+	*Vibrio* sp.
707-24	variable	-	rods, some long, similar to 707-15
707-25	-	+	Spirillum, *Aquaspirillum* sp.
707-26	-	+	identical to 707-5
707-27	-	-	similar to 707-1, thin rods
707-28	+	-	large irregular rods, *Gluconobacter* sp.

[1] Unless indicated as thin or fat, rods were 0.4 - 1.0 μm in diameter. Thin rods would be less than 0.4 μm and fat rods greater than 1.0 μm in diameter.

TABLE 26. CELL MORPHOLOGY AND TENTATIVE GENERIC ASSIGNMENT OF CINCINNATI WINTER ISOLATES (R3A BROTH OR SLANT)

Strain number	Gram stain	Motility	Cell morphology (R3A broth)[2]
2	-	-	long, medium-fat rods, inclusions
4	-	+	medium rods, some short rods
5	variable	-	long thin rods
8	-	-	coccobacillus
10	-	-	medium to long rods
14	-	+	large coccoid to egg-shaped rods, in pairs, Azomonas sp.
16	-	-	long, medium-width rods
21	-	+	like 14, Azomonas sp.
35	-	-	thin to fat pleomorphic rods
60	-	+	Aeromonas hydrophila
62	-	+	medium rods
4-3	-	-	irregular rods
4-4	-	+	coccobacillus
5-5[1]			medium to long rods
6-1	-	-	like 14, Azomonas sp.
6-2	-	-	long thin rods, clumps formed
6-5[1]	-	-	irregular rods of various lengths
6-7	-	-	medium rods
6-8	-	+	spirillum, Aquaspirillum
6-11[1]	Strain did not grow on subculture		
6-12[1]	Strain did not grow on subculture		
6-13[1]	-	-	fat rods
6-14	-	+	large rods
6-15	-	+	Aeromonas hydrophila
6-16	-	-	like 6-2

[1] These strains were lost (i.e., became non-viable) at some time prior to their complete characterization.

[2] Rods ranged in diameter from 0.4 - 1.0μm unless indicated as fat (greater than 1.0μm) or thin (less than 0.4μm).

Seattle system during the summer months Caulobacter spp. from one source, i.e. strains M3B1, M3A2 and M3A1, occurred in concentrations of 117,000 per ml and represented greater than 75% of the cultivable bacteria of that water sample. In that same survey other Caulobacter spp., K3A1 and K3A2, were found in a different chlorinated sample where they occurred in concentrations of 190 per ml which was in excess of 11% of the total viable count. It is interesting to note that not all Caulobacter spp. were identified by their morphology initially (Ref. Table 23) indicating the importance of observing wet mounts at the right time during growth and observing cells grown on an appropriate medium. Thus, K3A2 and M3B2 were discovered to be Caulobacter spp. at a subsequent period following the initial characterization. Likewise, though motility was not recorded as positive for these two strains, they were later found to be motile as are all species in the genus Caulobacter. Thus, if non-motility is recorded in these tables it may not be accurate, due to this problem. The winter strains of Caulobacter were I3 2/24 D1 and L3 2/24 D1.

Another prosthecate group, the Hyphomonas-Hyphomicrobium group can also be identified morphologically. These bacteria produce polar prosthecae (hyphae) and buds are produced at the tips of the appendages. Members of this group were frequently found in the Seattle distribution system and were isolated from chlorinated samples on several occasions. All strains were pink in pigmentation and appeared to be identical phenotypically. Colonies grew slowly appearing on DP isolation plates only after the usual 28-30 day incubation period. The strain used for characterization, I3K1, was the predominant organism from source I3 in the September 2, 1981 sample. At a concentration of 300/ml it comprised almost 60% of the viable count on that date. On the same date it occurred in another two chlorinated samples from other reservoirs, L3 and M3, at lower concentrations. It was found only in the Reservoir M3 sample on the November 12 sampling.

This strain resembles strains isolated from oligotrophic lakes in Australia (Staley et al., 1980) in its poor growth on rich media and pink pigmentation. This isolate appears to be a new species in the genus Hyphomonas (R. Moore, personal communication) and therefore will be labelled only as a Hyphomonas sp. in this investigation.

It is noteworthy that the Hyphomonas sp. occurred in both the reservoir samples and their chlorinated effluent (Table 27). From this observation it appears that it grew in the reservoirs rather than at subsequent points in the distribution system. This is an important point, since some members of this group can attach to surfaces and some have been reported in water pipelines and implicated in manganese deposition (Tyler and Marshall, 1973). When this strain was tested for manganese oxidation, it was negative. This preliminary evidence on this strain suggests it grew primarily, if not exclusively, in the reservoirs and not in the distribution system.

This Hyphomonas sp. appeared to survive chlorination as well as, if not somewhat better than, other strains from the reservoir samples. For example, in the Pre M3 reservoir it comprised only 2.8% of the viable bacteria in the September 2, 1981 sample compared to 10.0% of the viable bacteria in the chlorinated distribution water. On November 12, 1981 the percentages were

5.6% and 12.8%, respectively. In contrast, there appeared to be little change in the proportion of Hyphomonas sp. from L3 when compared to its reservoir (Pre L3) on September 2.

TABLE 27. CONCENTRATIONS OF HYPHOMONAS SP. IN IN-CITY RESERVOIRS AND CHLORINATED SAMPLES (DP AGAR)

		Reservoir			Chlorinated Sample		
Date	Location	Total heterotrophs/ml	Hyphomonas per ml	% Hyphomonas	Total heterotrophs/ml	Hyphomonas per ml	% Hyphomonas
Sept. 2, 1981	L3	160,000	10,000	6.2	3,100	170	5.5
Sept. 2, 1981	M3	400,000	11,000	2.8	160,000	16,000	10.0
Nov. 12, 1981	M3	480,000	26,600	5.6	180,000	22,600	12.8

Other Gram-negative bacteria were also morphologically distinctive. Perhaps the most notable of these were the vibrio and spiral shaped bacteria. Three spirilla strains were isolated. These were sent to an expert on the taxonomy of spirilla, Dr. Noel Krieg, at Virginia Polytechnic Institute. He has indicated that these are strains of Aquaspirillum. They are somewhat different from described strains in that they grow best as microaerophiles whereas known strains grow best as obligate aerobes. Thus, the species identification is uncertain. The strains included are 707-25, a summer isolate of Cincinnati, and 6-8 and 6-9, two winter isolates from Cincinnati. No spiral shaped bacteria were found in the Seattle chlorinated waters.

The motile vibrioid strain (707-23) from the Cincinnati summer isolates produces a black pigment (probably melanin) which is characteristic of certain species of Vibrio. It is sensitive to the 0/129 vibriostatic agent (5.0 mm radius inhibition zone), another characteristic indicative of this genus. The species has not been determined.

A few of the isolates were Gram-positive. Both Gram-positive rods and cocci were found. All of the Gram-positive cocci were obtained from the Seattle distribution system. Four of the five strains were isolated from the winter surveys. It was during these surveys that high levels of chlorine were used and very poor recoveries were obtained. Three of the strains (K3 11/12 A1, L3 2/24 A1 and L3 2/24 C1) were identified as Staphylococcus in that they fermented glucose on an O/F medium (original Hugh Leifson medium). One of the summer isolates (I3-D1) was a Gram-positive coccus which was pigmented yellow, formed packets, and did not ferment glucose. It was identified as Micrococcus luteus. The most unusual coccus, strain I3 2/24 G1, was characterized by motility, chain formation, and yellow pigmentation. It is possibly a strain of Planococcus, but further investigation would be needed to confirm this.

Only one of the Gram-positive rods was identifiable to genus. That was strain 707-28. It was able to grow at pH 4.5 and to produce acid from ethanol on Carr's medium, characteristics typical of the genus Gluconobacter. None of the other Gram-positive rods were similar to Gluconobacter spp. None were spore formers by the heat survival test used. Furthermore, none

grew anaerobically or fermented glucose, two characteristics expected of asporogenous Gram-positive rods such as found in the Lactobacillaceae. There were four such unidentifiable strains (strains I3-C1, L3-A1, L3-A2, and I3 2/24 H1). Likewise, two Gram variable rods, 707-21and 5, exhibited similar chacteristics and were not identifiable. With the exception of I3 2/24 H1 all were pigmented and non motile.

Another morphologically distinctive group was characterized by large ovoid, motile cells. These pink pigmented strains were all isolated from the Cincinnati winter collection and included strains 6-1, 14, and 21. These strains stained Gram-negative to Gram variable. Based upon morphological and physiological tests, these belong to the genus Azomonas.

The majority of the strains (72 of 102 strains tested) were Gram negative rods (Fig. 3). This included 36 strains from Cincinnati and 36 strains from Seattle. Considerable effort was directed toward the development of an appropriate test to determine whether the strains were fermentative. The Hugh-Leifson O/F test and several of its modifications were tested extensively with the hope of developing a satisfactory fermentation test medium for all strains. Unfortunately many of the isolates did not grow on this medium in its original formulation or in subsequent modifications that have been published. Also, anomalous results were frequently obtained, such as acid produced "fermentatively" by strains that were subsequently found to be obligate aerobes. Thus, this procedure was abandoned, and we developed a test to determine whether the bacteria could grow anaerobically. For this BBL Gas Pak containers were used and plates were streaked in pie sectors on a medium containing a readily fermentable sugar (glucose). Control plates were incubated aerobically. Only five of the Gram negative rods grew on the anaerobic plates: strains 2, 16, 35, 60, and 6-15. All of these strains were from Cincinnati. Two were identified by API-20E tests as Aeromonas hydrophila (60 and 6-15). The other three strains were not identifiable by the API-20E test kit. The source of these strains was not recorded so they may not have come from post chlorinated water in the distribution system.

We expected that a sizeable group of Pseudomonas strains would be recovered in this investigation. Thus procedures were used to distinguish organisms in this group. Phenotypically Pseudomonas sp. are Gram-negative, motile, obligately aerobic, generally non-pigmented, primarily oxidase positive, rods that can grow on a mineral salts medium containing acetate as sole carbon source (some would require vitamins and amino acids). Only one typical Pseudomonas sp. was encountered in the investigation. This strain was M3-C1; it was isolated from the Seattle summer survey and had all the features mentioned previously. Several strains of non-pigmented rods that were oxidase positive and motile resembled Pseudomonas species in all respects except for their ability to grow on a mineral medium with acetate as carbon source. Six of the Cincinnati strains, 6-14, 707-5, -9a, -9b, -12, -26, were of this type.

Twelve strains were obligately aerobic, oxidase negative, Gram-negative rods. Only two of these were able to grow on mineral acetate medium. These were pink-pigmented organisms from Seattle (I3 2/24 A1 and -A2). They were recorded as non-motile, otherwise they might be considered as strains of

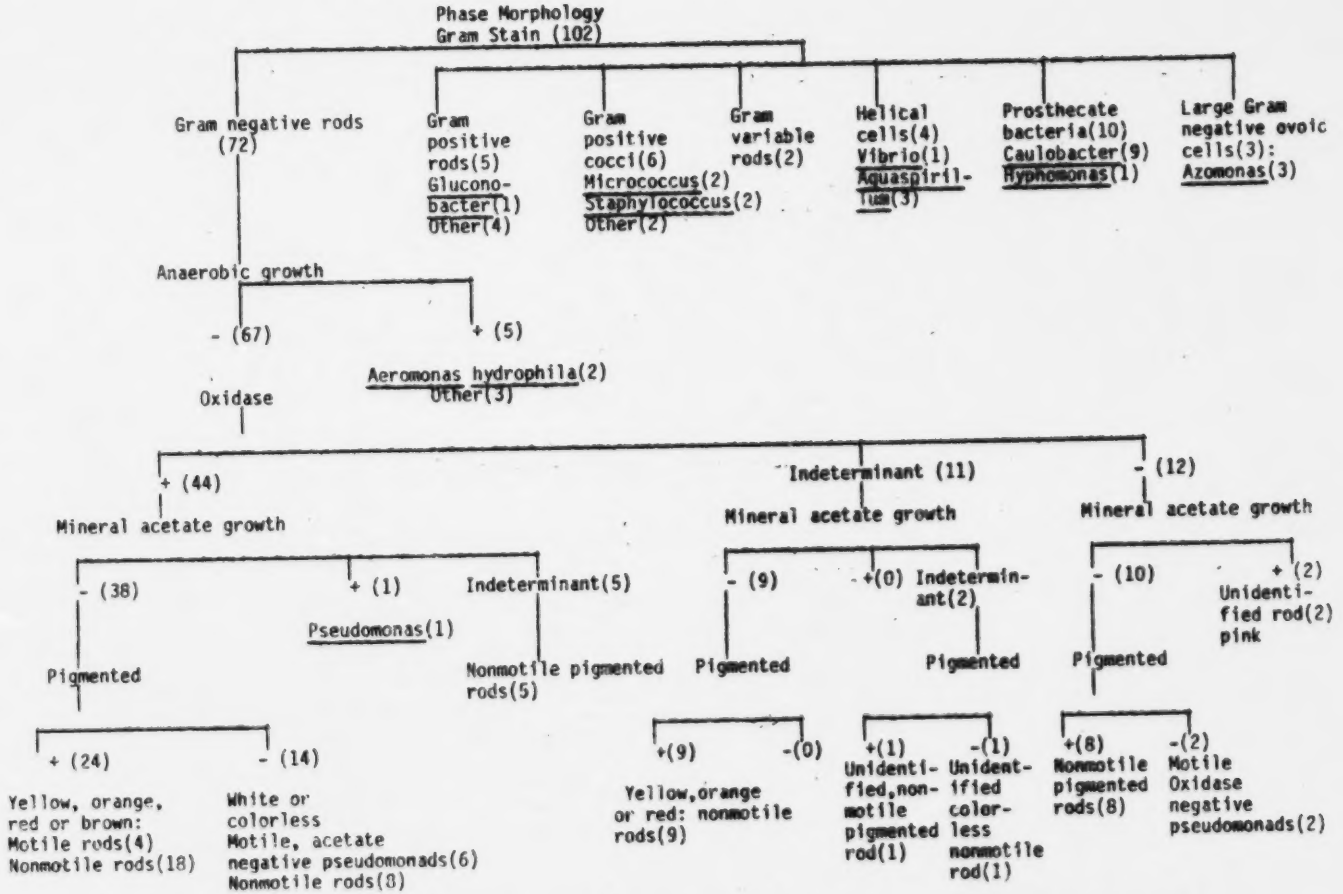

Figure 3. Flow diagram showing characteristics of 102 bacterial strains from chlorinated drinking water.

Pseudomonas, albeit oxidase negative ones. The other 10 strains in this group did not grow on mineral acetate. Only two of these were non-pigmented; these were 6-7 from Cincinnati and I3 2/24 F1 from Seattle, both isolated from the winter surveys. One of these strains was recorded as motile (6-7) but it is doubtful that it is a Pseudomonas sp. because it is unable to grow on mineral acetate and is oxidase negative. The other strain (I3 2/24 F1) digests agar on DP medium. The eight remaining strains were all pigmented and non-motile and included six Cincinnati isolates (4-3, 4-4, 6-2, 6-16, 707-1, -27) and two from Seattle (L3 2/24 G1, Myr 2/24 C1). These are possible candidates for the genus Flavobacterium (see Discussion).

The large majority of gram negative rods were oxidase positive. Of the 44 strains in this category, 23 strains were from Cincinnati and 21 strains were from Seattle. As has been mentioned previously one of these strains, M3-C1, was identified as a typical Pseudomonas sp. because of its lack of pigmentation, growth on mineral acetate medium, and motility. The remaining 43 strains included 38 that did not grow on the mineral acetate medium (Table 28) and five which were non-determinate with respect to growth on mineral acetate. All five of these latter strains were from Seattle, four from the summer survey (I3-E2, K3-B2, M3-D2, and L3-B1) and one from the winter (K3 11/12 A1). None of these strains was motile and all were pigmented. One of the summer strains was pigmented pink (L3-B1) whereas the others were pigmented yellow to yellow-brown.

TABLE 28. GRAM NEGATIVE, OXIDASE POSITIVE RODS THAT DO NOT GROW ON MINERAL ACETATE MEDIUM.

Pigmented strains
4*, 8, 10, 62*, 707-2, -8, -10, -11a, -17, -18, -24,
I3-A1*, K3-B1*, -D1, -G1, L3-C2, M3-D1,
I3 2/24 B1, K3 11/12 C1, -D1, M3 11/12 B1, -B3, Myr 2/24 A1
Nonpigmented strains
6-14*, 707-3, -4, -5*, -6, -7, -9a*, -9b*, 12*,
14, 15, 26*, K3-C1

* Motile

Of the 38 oxidase positive rods that failed to grow on mineral acetate medium, 24 were pigmented yellow, orange brown, brown or red. Only four of these were motile. The remaining 14 strains were non-pigmented (Table 28). Six of the non-pigmented strains were motile and, as mentioned previously, could be considered strains of Pseudomonas except for their inability to grow on the mineral acetate medium. All of these motile strains (6-14, 707-5, -9a, -9b, -12, -26) were from Cincinnati.

Eleven strains did not provide sufficient cell material on the non-carbohydrate medium to permit a conclusive oxidase test. All of these strains were from the Seattle distribution system, and with one exception (I3 2/24 C1), all were pigmented. The pigmented strains included I3-E1, -F1, K3-E1, L3-B2, I3 2/24 E1, K3 11/12 B1, M3 11/12 A1, -B2, -C1, and Myr 2/24 B1. The pigmentation varied from yellow to red and pink. The nonpigmented strain was nonmotile and indeterminant with respect to utilization of acetate. Thus, it is an unlikely candidate for the genus Pseudomonas.

Characteristics of the pigmented strains of the Gram negative rods are summarized in Table 29. Approximately half of these strains (i.e. 24 strains) were not able to grow at 37°C and some of these were unable to grow even at 30°C (seven strains did not grow at 30°C). Many of the strains did not grow sufficiently well to permit a thorough characterization. Thus, for example, although they might grow on the isolation and maintenance medium, it was not possible to obtain growth on the Hugh Leifson medium, or to obtain sufficient cell material for an oxidase test.

Three of the strains were able to grow anaerobically, but these did not produce growth on the Hugh Leifson O/F medium so that acid production from carbohydrates could not be determined. In contrast, some of the strains grew on the "anaerobic" Hugh Leifson medium and produced acid by "fermentation" even though they did not grow anaerobically. This indicates that the Hugh Leifson test is not only unsuitable but may be very misleading. When growth and acid production occurred by strains unable to grow in the Gas Pak anaerobic test, it was concluded that oxygen gradually diffused into the medium even though it might be covered with immersion oil or vaspar. The long incubation periods required for growth of these organisms made such tests questionable. Indeed, some isolates showed acid production "anaerobically" by the Hugh Leifson test but not aerobically. Such strains are likely microaerophiles.

Very few of the pigmented strains showed growth on the mineral acetate medium (three strains were recorded positive) and a few were able to utilize beta-hydroxybutyric acid as a carbon source (nine strains). Only one was able to grow at pH 4.5.

Though not recorded in this table, a number of features were tested and found to be universally negative for the pigmented strains shown in the table. None survived the heat survival tests for spore formers. Likewise, none was able to digest cellulose as a carbon source, a distinctive feature of the genus Cellulomonas, a group of pigmented Gram-negative rods.

Discussion

TABLE 29. CHARACTERISTICS OF PIGMENTED GRAM NEGATIVE RODS

Strain	Color	Growth Temperature (°C) 20-25	30	37	Oxidase	Anaerobic Growth	O/F (Glucose)	PHB Utilization	Growth pH 4.5	Growth on mineral acetate
6-2	yellow	+	+	+	-	-	NG[1]	-	-	-
6-16	yellow	+	+	+	-	-	NG	-	-	-
2	yellow	+	+	+	+	+	NG	-	-	-
4	yellow	+	+	+	+	-	NG	-	-	-
8	yellow	+	+	+	+	-	NG	-	-	-
10	orange-brown	+	+	+	+	-	NG	-	-	-
16	yellow	+	+	+	+	+	NG	-	-	-
35	orange-brown	+	+	+	-	+	NG	-	-	-
4-3	orange-brown	+	+	+	-	-	NG	-	-	-
4-4	yellow	+	+	-	-	-	NG	-	-	-
707-1	yellow	+	+	+	-	-	A/A[2]	-	-	-
707-2	orange	+	+	+	+	-	K/NC	-	-	-
707-8	orange	+	-	-	+	-	K/NC	-	-	-
707-10	yellow	+	+	+	+	-	K/NC	+	-	-
707-11a	yellow	+	+	-	+	-	K/NC	+	-	-
707-17	orange	+	+	+	+	-	K/NC	-	-	-
707-18	yellow	+	+	+	+	-	K/S1A	+	+	-
707-24	orange	+	+	-	+	-	K/NC	+	-	-
707-27	yellow	+	+	-	-	-	S1A/NC	-	-	-
I3 E1	yellow	+	N.D.[3]	N.D.	N.D.	-	NC/NC	-	N.A.	-
I3 E2	yellow	+	N.D.	N.D.	+	-	N.D.	N.D.	N.D.	N.D.
I3 F1	yellow-orange	+	N.D.	N.D.	N.D	-	NC/NC	-	N.D.	-
K3 B1	yellow-orange	+	+	-	+	-	K/NC	+	N.D.	-
K B2	yellow-orange	+	N.D.	N.D.	+	-	N.D.	N.D.	N.D.	N.D.
K3 E1	light yellow	+	+	-	N.D.	-	A/NC	-	-	-
L3 B1	purple	+	+	-	+	-	NG	NG	-	N.D.
L3 B2	purple	+	+	-	+	-	NG	-	-	N.D.
K3 D1	pink	+	+	-	+	-	K/NC	+	-	-
K3 G1	yellow	+	+	-	+	-	K/NC	-	-	-
L3 C2	yellow	+	-	-	+	-	A/NC	-	-	-
M3 D1	yellow	+	+	N.D.	+	-	NC/NC	-	-	-
M3 D2	yellow	+	N.D.	N.D.	+	-	N.D.	N.D.	N.D.	N.D.
I3 2/24 A1[5]	pink	+	+	+	-	-	K/NC	+	-	+
I3 2/24 A2[5]	pink	+	+	+	-	-	K/NC	+	-	+
I3 2/24 B1	yellow	+	+	-	+	-	A/A	-	-	-
I3 2/24 E1	yellow	+	-	-	N.D.	-	NG	-	-	-
K3 11/12 D1	yellow-orange	+	+	-	+	-	NC/S1A[4]	-	-	-
M3 11/12 A1	tan	+	-	-	N.D.	-	NC/NC	-	-	-
M3 11/12 B1	tan	+	+	-	+	-	NC/S1A	-	-	-
M3 11/12 B2	tan	+	-	-	+	-	NC/S1A	-	-	-
M3 11/12 B3	tan	+	-	-	+	-	NC/S1A	-	-	-
M3 11/12 C1	purple	+	+	-	N.D.	-	NG	-	-	-
Myr 2/24 A1	brown	+	-	-	+	-	NC/NC	-	-	-
Myr 2/24 B1	yellow	+	+	-	-	-	A/A	-	-	-
Myr 2/24 C1	yellow	+	+	-	-	-	A/A	+	-	-
L3 2/24 G1	yellow	+	+	+	-	-	N.D.	NG	NG	NG
L3 2/24 G1	yellow	+	+	+	-	-	N.D.	NG	NG	NG
K3 11/12 A1	yellow	+	N.D.	N.D.	+	-	N.D.	N.D.	N.D.	N.D.
K3 11/12 B1	yellow	+	-	-	N.D.	-	NG	-	-	-
K3 11/12 C1	yellow	+	N.D.	N.D.	+	-	NG	-	-	-

1 NG - No growth.
2 A/A - Acid aerobically/acid anaerobically; K - alkaline aerobically; NC - no change; S1 A - slight acid.
3 N.D. - Not determined.
4 NC/S1A - This O/F reaction suggests organisms are microaerophilic and obtain some air with prolonged incubation.
5 Colorless on King^A medium but pink on maintenance media.

A number of interesting results have been obtained in the investigation. One discovery was that of finding appreciable numbers of prosthecate bacteria of the genera Caulobacter and Hyphomonas in drinking waters. Though these have been known as aquatic bacteria for many years there have been no publications of which we are aware, that report their occurrence in chlorinated drinking waters. The genus Caulobacter, in particular, appears to be widespread in its distribution since strains were obtained both from Cincinnati as well as Seattle, and winter as well as summer isolates have been obtained. Hyphomonas was found only in the Seattle system, but appeared to be a significant member of the community, as it was found in more than one of the reservoirs and was found at high concentrations at certain times. Likewise Caulobacter spp. can comprise a significant proportion of the bacteria in drinking waters. Perhaps these prosthecate bacteria may be enriched in systems that have reservoirs with 5-30 day detention times that permit regrowth to occur.

It was also of interest to note that spiral shaped bacteria in the genera Aquaspirillum and Vibrio were detectable in the Cincinnati water distribution system. Three of the 50 isolates characterized from Cincinnati were strains of Aquaspirillum, so it appears to be an important component of the heterotrophic bacterial flora of drinking waters from that distribution system.

The discovery of a strain of Vibrio sp. was most surprising since many species of this genus are pathogenic to humans or fish. It would be of interest to identify this particular black pigmented strain as it may possibly be a fish pathogen (we have given this strain to Dr. Jorge Crosa as he is interested in taxonomy of the genus; he has indicated that some black pigmented strains are fish pathogens; however, we have not yet learned its identity).

Another rather distinctive morphotype, based on cellular features, was a group of three large ovoid Gram-negative to Gram-variable rods. These three strains were pink in pigmentation, motile, and all were recovered from the Cincinnati distribution system. Based upon carbon source utilization patterns, these were classified as Azomonas spp; they differed from described species in pigmentation and carbon source utilization patterns.

Gram-positive cocci were occasionally encountered, particularly during the Seattle winter survey when very high levels of chlorination were encountered. Two of the six strains in this category were identified to the genus Staphylococcus and two to the genus Micrococcus; the remaining two were atypical.

The final group of morphologically distinctive bacteria were Gram-positive to variable rods. Of the seven strains in this category only one was identified to genus. It was a member of the genus Gluconobacter, one of the acetic acid bacteria. The remaining organisms in this group were oxidase positive, non-fermentative, and with one exception all were non-motile and pigmented.

The majority of the isolates (72 of 102) were gram negative rods. Most of these (67 of 72) were obligate aerobes unable to grow fermentatively on complex media containing glucose as a carbon source. Two of the facultative anaerobic fermentative rods were identified as Aeromonas hydrophila on the basis of their API-20E test patterns.

Of the 67 strains of obligately aerobic rods only one was clearly identifiable as a member of the genus Pseudomonas. This strain was oxidase positive, motile, non-pigmented, and grew on a mineral acetate medium recommended for the growth of Pseudomonas spp. A few other motile Gram negative rods (twelve strains altogether) differed from the typical Pseudomonas characteristics in one or two major features such as (a) inability to grow on mineral acetate medium (six strains), (b) oxidase negative and inability to grow on mineral acetate medium (two strains), or (c) pigmentation and inability to grow on mineral acetate medium (four strains).

Almost half of the strains studied in this survey (49 of 102) were pigmented, obligately aerobic Gram-negative rods. They differed from one another in terms of their growth on mineral acetate, oxidase reaction, and color of pigmentation. Only four were identified as motile. The largest group (18 of 49) had the single phenotype of: oxidase positive, mineral acetate growth negative, and nonmotility.

Because the pigmented Gram-negative rods comprised an especially large group of the isolates and were well represented by both sources of isolates, much effort was placed in their identification. Genera listed in the eighth edition of Bergey's Manual which contain Gram-negative heterotrophic pigmented rods include Pseudomonas, Flavobacterium, various myxobacteria, various cytophagas, Arthrobacter, Cellulomonas and Xanthomonas. The gliding groups can be eliminated because the isolates we have studied are not motile by gliding and do not show the other features typical of these organisms. Cellulomonas can be eliminated because none of the isolates grew on cellulose in comparison with known strains of the genus. Xanthomonas spp. and Pseudomonas spp. are motile, whereas few of our pigmented isolates have been found to be motile. Arthrobacter spp. may be pigmented, but these should stain as Gram-variable, have coccus stages, and be motile, uncharacteristic features for our isolates.

It is possible that some, perhaps even many, of these pigmented Gram-negative rods could be classified as strains of the genus Flavobacterium as the genus Flavobacterium is defined (Eighth edition of Bergey's Manual) so broadly that it encompasses virtually all pigmented Gram-negative rods. Thus, both nonmotile and motile forms are present, and although the genus description indicates that only flagellated types are included, F. aquatile is described as having a gliding type motility. Likewise, though most organisms in the group are respiratory and obligately aerobic, some are described as facultative anaerobes. Pigments are insoluble and presumably carotenoid, although pigment type has oftentimes not been determined. Colors include yellow, orange, brown, and red. In addition, both low DNA base composition strains (25-35 moles % G+C) as well as high DNA base composition strains (65-70%) are included in the genus. Flavobacterium is a heterogenous collection of organisms. It can be regarded as a virtual "wastebasket"

containing a broad diversity of pigmented Gram-negative rods, many of which are very different from others. Though it could be considered an appropriate place to include the isolates of this investigation we are reluctant to do this without further evidence for three primary reasons. First, we have no convincing evidence our strains are related to those that are currently placed in the genus. Second, we have found some nonpigmented strains that closely resemble the pigmented groups. It is quite possible that these are more closely related to the pigmented strains than to Pseudomonas spp. because they are nonmotile and most do not grow on mineral acetate medium. If they are really closely related to the pigmented strains, then they could not be included in the genus Flavobacterium because they are non-pigmented. This would seem irrational to us as pigmentation often varies within a genus. Third, the group of pigmented strains found in this study is very diverse indeed. It is doubtful that all members could be placed in a single genus based upon the wide range in phenotypic characteristics. For example, some strains were recorded as Gram-positive rods (L3 A1 and L3 A2) whereas most are Gram-negative. Some are oxidase positive, some oxidase negative, and some indeterminant with respect to this property. Some are motile, others are nonmotile. Some are facultative anaerobes whereas most are respiratory. Some grow on mineral acetate, others do not and some are indeterminant with respect to this characteristic. I know of no single genus that would contain such diversity, including even the genus Flavobacterium, as it is currently defined.

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